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IMPROVED DIAGNOSIS AND VACCINATION OF PET ALLERGY - FROM MOLECULAR MECHANISMS TO CLINIC

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Improved diagnosis and vaccination of pet allergy - from molecular mechanisms to clinic

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“There is a theory which states that if ever anyone discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable.

There is another theory mentioned, which states that this has already happened”

Douglas Adams

To Molly and Ellis

ABSTRACT

Allergy is a disease affecting around 30% of the population worldwide, causing extensive suffering for individual patients and constituting a significant socioeconomic burden to society. The immunological reaction in allergy causes symptoms in the range from mild itching and runny nose to systemic death threatening states. One common cause of allergy is pets, such as dog and horse. Today's diagnostics of pet allergy is frequently based on extract from dander or hair, however extracts may vary in content of allergens. The only curative treatment is allergen specific immunotherapy (SIT), routinely performed by injections with allergen extracts. For both diagnostics and treatment, the content of allergen component in the extracts is vital. The application of DNA technology has opened opportunities to produce allergens in pure form and at good yield, making the allergen components available for both diagnostics and SIT. Further improvement of SIT could be achieved by the use of adjuvants able to skew the allergic immune response to a non-allergic response. Currently, the adjuvant alum is used in SIT. New adjuvants are needed that more efficiently stimulate regulatory or Th1 type responses.

The **aim** of this thesis was to identify and analyze new sources of pet allergens, to identify new allergen components from horse and dog, and to investigate mechanisms and clinical safety/efficacy of a novel adjuvant candidate based on chitosan, which will possibly be suitable for future use in SIT, as well as for other vaccine applications.

In **paper I** of this thesis we investigated dog saliva as a possible allergen source, and if today's diagnostic extract could be improved by using saliva. We found that some individuals with negative IgE test to dog dander have IgE to dog saliva, and that the IgE binding is biologically relevant, as dog saliva could activate basophil degranulation. Using immuno-proteomic analyses, four potential IgE binding proteins not previously described as allergens were identified.
In **paper II** we present a thorough analysis of horse allergen components. We identified three novel full-length IgE binding proteins and evaluated the prevalence of IgE reactivity among 100 sera from horse sensitized individuals. All three novel allergens belong to protein families from which allergens from dog, cat or cow have previously been described. The prevalence of sensitization to the new allergens ranged between 34 and 66% and together with three additional known horse allergens all 100 sensitized individuals could be detected.
Paper III investigated the adjuvant candidate ViscoGel, composed of chitosan based viscoelastic particles. ViscoGel's ability to be phagocytosed by, and activate antigen presenting cells was studied *in vitro*. The antigen presenting cells were able to take up the chitosan particles of 10 and 200µm size, and to stimulate the release of IL-1β in a caspase-1 independent manner.
Paper IV describes a clinical phase I/IIa trial evaluating safety of ViscoGel alone and in combination with the model vaccine Act-HIB, and efficacy as an adjuvant for Act-HIB. ViscoGel was well tolerated when injected intramuscularly. No adjuvant effect was observed on the antibody response to Act-HIB, but the IFNγ response was affected, suggesting that ViscoGel may promote a Th1 type of response.

To conclude, the results presented in this thesis have the potential to improve diagnostics of allergy to dog and horse. Moreover, a new potential adjuvant was shown to be safe and exhibited immunological properties that may be favorable for use in SIT.

LIST OF SCIENTIFIC PAPERS

I. Polovic N*, Wadén K*, Binnmyr J*, Hamsten C, Grönneberg R, Palmberg C,Milcic-Matic N, Bergman T, Grönlund H, van Hage M.
Dog saliva - an important source of dog allergens.
Allergy. 2013;68(5):585-92
*these authors contributed equally

II. Binnmyr J,Holmgren H, Bronge M, Gafvelin G, Grönlund H
IgE profile in horse (Equus caballus) sensitized subjects using novel and described allergens
Manuscript

III. Binnmyr J, Olliver M, Niemert-Andersson T, Heidenvall S, Vukojevic V, Grönlund H, Gafvelin G.
Uptake of chitosan based hydroelastic particles in vitro by antigen presenting cells and activation of innate immune responses
Submitted

IV. Neimert-Andersson T*, Binnmyr J*, Enoksson M*, Langebäck J, Zettergren L,Hällgren AC, Franzén H, Lind Enoksson S, Lafolie P, Lindberg A, Al-Tawil N,Andersson M, Singer P, Grönlund H, Gafvelin G.
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Allergy. 2013 Nov;68(11):1485-6

CONTENTS

LIST OF ABBREVIATIONS

Act-HIB	Haemophilus influenzae type b vaccine
AE	Adverse event
α-Gal	galactose-α-1,3-galactose
Alum	Aluminium hydroxide
AIM	Absent in melanoma
APC	Antigen presenting cells
BAT	Basophil activation test
CARD	Caspase activation and recruitment domain
CD	Cluster of differentiation
CTL	Cytotoxic T lymphcyte
DAMP	Damage-associated molecular pattern molecules
DC	Dendritic cells
ELISA	Enzyme linked immunosorbent assay
HDE	Horse dander extract
HDE-e3	Commercial HDE on ImmunoCAP
HIB	*Haemophilus influenza* type B
HLA	Human leukocyte antigen
HSP	Heat shock proteins
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMAC	Immobilized metal chelating chromatography
ILIT	Intralymphatic immunotherapy
IRF	Interferon regulatory factors
ITAM	Immunoreceptor tyrosin-based activation
LPS	Lipopolysacharide
LRR	Leucine rich repeats
MAPK	Mitogen associated protein kinase
MD2	Myeloid differentiating factor 2

MDDC	Monocyte derived DCs
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation factor 88
NAIP	Neuronal apoptosis inhibitory proteins
MS/MS	Tandem mass spectrometry
NLR	NOD-like receptor
NLRP3	Nucleotide-binding oligomerization domain (NOD) NOD-like receptor and pyrin domain containing protein 3
NFκB	Nuclear factor kappa light-chain enhancer of activated B-cells
NOD	Nucleotide-binding oligomerization domain
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SIT	Allergen specific immunotherapy
SPT	Skin prick test
TCR	T-cell receptor
TIR	Toll/Interleukin-1 receptor
TLR	Toll-like receptor
Treg	T regulatory cell
TRAF	TNF receptor associated factor
TRIF	TIR-domain-containing adapter inducing interferon-B
TT	Tetanus toxoid
OD	Optical density
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PMA	Phorbol 12-myristate 13-acetat
PRR	Pathogen recognition receptors

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The human body has been fighting different diseases and harmful pathogens in our environment throughout evolution. These efforts, on individual basis, are made to maintain the integrity of our bodies intact, preventing cells from dying and preserving nutrients for ourselves (1). This has driven us into evolving an immune system with a wide range of mechanism to both escape and attack the potentially harmful agents. Usually this is the classical way to consider what function the immune system has, attacking foreign molecules that can potentially harm us and neglect own-produced molecules necessary for survival. Somewhat contradicting to this picture to discriminate between self and non-self, is the immune system's ability to recognize infected cells or even tumor cells (2). Therefore the route by which activation of the immune system occurs is complex, with different results dependent on the activation signal.

To protect against pathogens, the human body is covered with a protective barrier, the skin. The skin's epithelial layer is hard to penetrate for any bacteria or virus. It is also covered by non-pathogenic microorganisms, a microbiota that will compete with potential pathogens for both space and nutrients. The skin is coated with proteins and anti-microbial peptides, targeting bacteria, adding to the outer defense against pathogens (3, 4). The outer layer of the skin is just the first line of defense that a pathogen has to overcome to infect a human.

The multifaceted world of proteins and cells that fight against our enemies is usually divided into to two specific systems, innate immunity and adaptive immunity (5). The innate system is the quick responder that we are born with, it is inherited and highly conserved, reacting towards conserved recognition patterns derived from pathogens (6). The adaptive immunity is on the other hand constantly evolving, modulating its receptors for pathogen recognition. Therefore it is slower in the start, however vastly more specific and with the ability to undergo expansion. Not only does it react but it is also capable of creating a memory of the pathogen, making the next encounter of the same pathogen easier to react toward, both faster and more specific than the initial response.

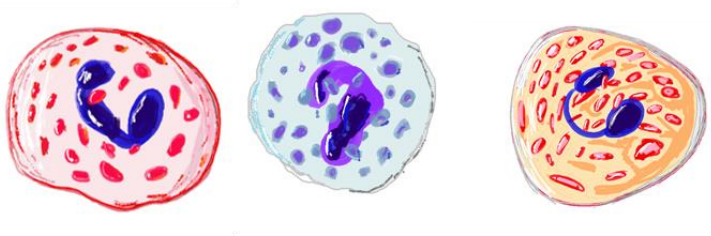
1.2 ANATOMY OF THE IMMUNE SYSTEM

To give a perspective of the important anatomical structures and organs of the immune system, one should first consider the barriers pathogens need to penetrate. That is the skin—or in Latin *epidermis*— for the exterior part of the body, and the interior is the mucus membrane—in Latin the *mucosa*. These barriers are covered by physical obstacles preventing penetration, such as dead cells, bacteria and mucus. Epithelial cells are not immunological cells per se, but are still important both as a barrier, and relevant for this thesis, as a mediator of signaling in inflammation and disease (7). The barriers, which the epithelium make up, need to signal to immune cells which state it is in and induce and facilitate migration of cells into the tissue, so they have a vital role in the immunological interaction, and also play a role in skewing the response (8). Other structures important for the immune system are the bone marrow, from where the stem cells of the blood system originate. The process when blood cells mature from a stem cell is called the hematopoiesis, and is the origin of most immunological cells, with a few exceptions. The bone marrow together with the thymus make up what is called the primary lymphoid organs. In the thymus a sub-set of immune cells undergo negative selection based on their recognition of self-

molecules, to prevent reactions to own tissue. Secondary lymphoid organs are the lymph node and the spleen, where some immunological cells are activated, based on their recognition of foreign molecules, and where the communication between the innate and the adaptive immune system occurs.

1.3 CELLS OF THE IMMUNE SYSTEM

Most cells that belong to the immune system are cells stemming from a common pluripotent stem cell that resides in the bone marrow, but there are exceptions and still cells with unknown progenitors and unclear anatomical origin (7). The bone marrow derived cells migrate into the bloodstream, where they are patrolling and ready to migrate into stress- or danger-signaling tissue. Dependent on their site of activation and maturation they are divided into myeloid or lymphoid cells, where the myeloid cells belong to the innate immunity and lymphoid cells belong to the adaptive, but there are exceptions from this distinction of cells as well. To identify and discriminate between different cells a system called cluster of differentiation (CD) has been developed to classify surface expressed molecules (9)



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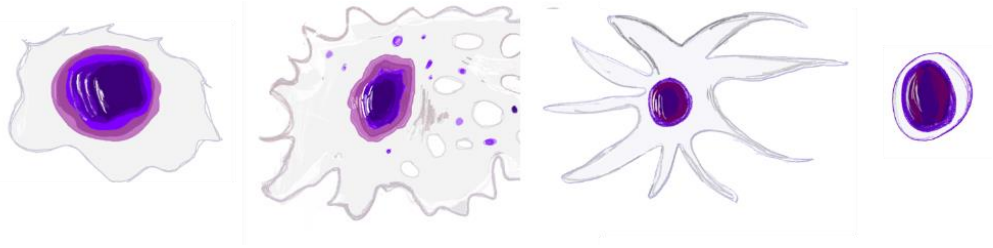
Figur 1 **Granulocytes** From left to right: neutrophil, basophil and eosinophil.



Figur 2 **ANTIGEN PRESENTING CELLS AND LYMPHOCYTES** From left to right: monocyte, macrophage, DC and lymphocyte.

1.3.1 **Granulocytes**

Three subtypes of cells packed with vesicles, filled with effector molecules and mediators, named after their granules are identified granulocytes (10). They are colored differently when stained with hematoxylin and eosinophilic stain, and the circulating subclasses are named after their staining: basophils, neutrophils and eosinophils (figure 1).

1.3.1.1 Neutrophils

Neutrophils are abundant in the blood and infiltrate inflamed or damaged tissue within just a few hours (11). They are characterized by a polymorphic nucleus and are phagocyting cells that can release neutrophil extracellular trap, to trap pathogens. Neutrophils also have the ability to migrate to lymph node upon activation and function as a hybrid between dendritic cell (DC) and neutrophil (12, 13).

1.3.1.2 Basophils

Basophils are rare cells, only around 1% of the granulocytes are basophils. However, they are important cells of allergic reaction (14). Basophils have the ability upon cross-linking of IgE bound to the high affinity receptor of IgE (FcεRI) to release histamine and other mediators, Together with mast cells they are key players in the early phase of the allergic reaction. Since basophils are present in the blood, they may be used in the clinical evaluation of allergic reactions (15, 16). Basophils and mast cells can release the allergy associated mediator histamine, proteases, cytokines and other inflammatory mediators.

1.3.1.3 Eosinophils

Eosinophils, like neutrophils, are rapidly recruited to inflammatory sites (17). They have been described to play a role in the defense against parasites but also in the development of asthma (18). The eosinophil, as the other granulocytes, release their granulae content upon activation. Eosinophils release inflammatory mediators and proteins that propagates and modulates the immunological reaction.

1.3.1.4 Mast cells

Mast cells are tissue resident cells, which are major players in allergic disease (19). They share many features of the basophil, including expression of the high affinity IgE receptor, ability to release histamine and other mediators upon allergen mediated cross-linking of IgE receptors and activation of other immunological cells (19).

1.3.2 **Antigen presenting cells**

The ability to take up extracellular molecules and present these to the adaptive immune system is the function of antigen presenting cells (figure 2)(APCs)(20). These cells include DCs and macrophages, which belong to the innate immunological cells, and the B-cells (the latter will be addressed under the description of adaptive immune cells). Common for the APCs are their ability to present foreign peptides. However, new insights make this classical nomenclature disputable, with reports of granulocytes acting as APCs (13, 20). Therefore one discriminate between professional, i.e. B-cells, DCs and macrophages, and atypical, i.e. all other cells with unclear ability to induce T lymphocytes.

1.3.2.1 Antigen presentation.

There are two classes of the major histocompatibility complex (MHC) molecules—class I and II—which are encoded by distinct clusters of genes (21). They present peptide fragment from proteins, either from cytosolic derived proteins (MHC I), or exogenous proteins found in intracellular vesicles (MHC II) (22). MHC I molecules on APCs have however also been demonstrated to present peptides derived from proteins taken up by the cell, a process called cross-presentation (23). They become loaded on the MHC I on professional APCs and are then transported to the cell-surface with the peptide attached. The MCH-peptide complex is then presented to the T-cell via the T-cell receptor (TCR). Professional APCs have a special role in antigen presentation, they have the ability to activate naive T-cells via co-stimulatory molecules on their surface. In humans, the genes encoding MHC I and II are called human leukocyte antigen (HLA), the A, B and C type encodes the MHC I and the HLA-DR, DQ and DR encodes the MHC II (23).MHC I presents peptides of 8-9 amino acids in length and MHC II peptides of15-24 amino acids (22).

1.3.2.2 Monocytes

Monocytes are mononuclear cells patrolling the bloodstream. They migrate to sites of inflammation and infection, via blood vessels. There the monocytes develop into macrophages or DCs dependent on the signaling from the surrounding (24).

1.3.2.3 Macrophages

Macrophages are professional phagocytizing cells. There are two distinct macrophage subtypes, M1 and M2, distinguished by their ability to produce IFNγ and nitric oxide (M1), or to promote tissue repair and produce interleukin (IL)-6 (M2) (25). Macrophages belong to the professional APCs. They express pathogen recognition receptors like Nucleotide-binding oligomerization domain (NOD)-like receptors (NLR), and are key cells in microbial defense. Moreover, as APCs they have the ability to present antigen to T-cells via MHC class II as well as MHC I. Macrophages could be derived from monocytes or be tissue resident (24).

1.3.2.4 Dendritic cells

Dendritic cells have a specific role in the induction of the adaptive response(26). Upon activation of DCs, usually due to infection or inflammation, the cells will migrate to the lymph node and present peptides from the periphery to antigen specific T lymphocytes and activate them. Dendritic cells both express MHC class I and II and upon activation they expresses co-stimulatory molecules, making T-cell activation and priming of an adaptive response possible (27). In addition, molecules secreted from the DCs contribute to the shaping of the immune response by inducing functionally distinct effector cells (27).

1.3.3 **Lymphocytes**

Cells found in the lymph system are referred to as lymphocytes and are classified dependent of function (figure 2)(7). The lymphocytes described in this thesis are the cells of the adaptive immunity, however some subclasses of lymphocytes or lymphoid cells belong to the innate immunity, e.g. NK cells, NKT cells and innate lymphoid cells (7).

1.3.3.1 T-cells

T lymphocytes are named after the location where they undergo selection of their antigen binding capacity, namely the thymus (7). They belong to the adaptive immunity and can be divided into several different subclasses. T-cells are identified via the cell-surface protein CD3, an accessory protein acting as a co-receptor to the TCR, responsible for antigen recognition. The TCR undergoes selection in the thymus based on the ability to bind MHC molecules, were T-cells with TCR that do not bind or binds to strong is sorted out and undergo apoptosis (28). The T-cells that pass this selection enter the circulation and start patrolling the periphery until they encounter an APC that presents an a peptide in the context of an MHC which is recognized by the T cell together with a co-stimulatory signal (28, 29). Subclasses of T-cells include the CD8 positive T-cells, or cytotoxic T-cells (CTL), which are specialized in killing cells. The CTLs present antigen on MHC class I and killing is mediated by grancymes or FAS ligand interaction with the MHCI presenting cells (30). CD4 positive cells, or T helper cells (Th), are promoting humoral immune responses. There are several Th

subclasses, the first two identified were Th1 and Th2 (31). The subclasses are identified by their cytokine signatures or expression of transcription factors; Th1 cells release IFNγ and expresses T-bet and Th2 cells releases IL-4, IL-5 and IL-13 and expresses GATA3 (32, 33). Later, a number of Th subclasses have been described, the most established being Th17, expressing RORγt, and T regulatory (Treg) cells. Th17 cells secrete the signature cytokine IL-17, while Treg cells may secrete immune regulatory cytokines such as IL-10 and TGF-β and is usually identified by the transcription factor FOXP3 (31, 34-36). Each Th subclass is associated with different immunological responses; Th1 mediate responses against intracellular pathogens, Th2 against extracellular parasites and Th17 against extracellular bacteria. The function of Tregs is to down-regulate immune responses or to induce tolerance.

1.3.3.2 B-cells

B-cells are named after the organ where the cells were first identified — the bursa of birds— a lymphoid organ localized in close proximity to the gut of birds (37). However the name of the cells can also be applied on their origin in mammals, the bone-marrow. B cells belong to the adaptive immunity (20). They start their development in the bone marrow where the B cell undergo selection based on the rearrangement of immunoglobulin (Ig) genes (38). The B-cells then becomes activated once the cell has taken up foreign protein via its cell-surface bound Ig, internalized it, degraded and presented it on MHC II to an activated Th-cell (7, 29, 39). This leads to induction of the B-cell to produce soluble Ig, followed by clonal expansion, Ig sub-class switching and establishing immunological memory (39). However, B-cells can start producing Igs independent of T-cells, a process where repetitive epitopes, such as carbohydrates, binds and activates the B-cell without T-cell help (40). In addition to their role as antibody producers, B-cells can also act as professional APCs (20)

1.3.3.3 Immunoglobulin

Immunoglobulin—or antibodies— are proteins produced by B-cells, specialized in binding antigens. The ability of antibodies to generate immunity against pathogens and toxins was discovered in the late 1800. There are five different Ig subclasses found in humans: IgA, IgD, IgG, IgE and IgM. Each Ig subclass has a specialized function (37, 41). The structure, with two heavy chains and two light chains, can be visualized as the shape of a Y (41). The antibodies consist of a variable region at the "tip" of the Y, which binds to the antigen, and a constant region, with the ability to bind to cellular antibody Fc receptors dependent on the class of the constant region (41). For example, and relevant for this thesis, the IgE antibody subclass binds the high affinity receptor FcεRI and the low-affinity receptor FcεRII (16). The high affinity receptor is expressed mainly on mast cells and on basophils, and the low affinity receptor is expressed , among others, by B-cells (16). One could view this cellular binding of Ig:s to Fc-receptors as a way of the adaptive immunity to transfer its antigen recognition to specialized effector cells.

1.4 IMMUNOLOGICAL RECEPTORS OF ACTIVATION.

To activate cells to target pathogens or in response to stress, cells display and produce a broad variety of molecular detectors (42). These receptors, or pathogen recognition receptors (PRR), are activated by different classes of ligands. They may be further divided into different subclasses,

called pathogen associated molecular patterns (PAMP) or Damage-associated molecular pattern molecules (DAMP), dependent of the source of activation.

Table 1 **Toll-like receptors** the ten toll-like receptors described in humans.

	Toll-like receptors		
	Pathogen Ligands	Endogenous Ligands	Cellular location
TLR1/2	Triacyl lipopeptides	-	Cell membrane
TLR2/6	Diacyl lipopeptides, zymosan	HSP	Cell membrane
TLR3	dsRNA	mRNA	Endosome
TLR4	LPS	HSP	Cell membrane
TLR5	Flagelin	-	Cell membrane
TLR7	ssRNA	immune complexes	Endosome
TLR8	ssRNA	immune complexes	Endosome
TLR9	CpG-DNA	immune complexes	Endosome
TLR10	-	-	-

1.4.1 **Toll-like receptors.**

Among the well-studied innate receptors are Toll-like receptors (TLR) (German for funny-looking receptors) that are characterized by an N-terminal leucine rich repeats (LRR) domain and a cytoplasmic Toll/IL-1 receptor (TIR)-domain (43-48). These receptors all react towards different types of ligands. Ten different TLRs have been identified in humans (Table 1)(covered in this thesis) (48). TLRs signal via the myeloid differentiation primary response gene 88 (MyD88) or TIR-domain-containing adapter inducing interferon (IFN)-B (TRIF) signaling pathway. They can be divided into different clusters dependent on their ligands: some react towards lipids, other against bacteria or virus derived molecules. TLR1 and TLR 6 form a dimer with TLR2, and are cell membrane bound receptors (figure 3)(48). The TLR1/2 dimer reacts towards lipoproteins, but TLR2/6 are broader in activation, with both recognition of microbial ligands such as lipopeptides and zymosan, but also have endogenous activators such as heat shock proteins (HSP) (47). When bound to their ligands, these TLRs activate MyD88 downstream (figure 3). TLR3 is not found on the cell surface, but expressed on endosomes (figure 4) and does not activate MyD88. Instead it activates the TRIF pathway, in response to its virus derived ligand dsRNA (6). TLR4 was discovered via a spontaneous mutation in the mouse gene lps, making these mice tolerating high levels of endotoxins (49). TLR4 recognizes lipopolysaccharide (LPS) in combination with the protein myeloid differentiating factor 2 (MD2) (50, 51). TLR4 is the only TLR that activates both the MyD88 pathway and the TRIF pathway and can be internalized, therefore it works both as a surface-receptor and an endosome receptor (figure 3). TLR5 is activated by bacteria-derived flagelin and is also expressed on the cell surface and activates the MyD88 pathway (figure 3) (6). TLR7 and 9 resides in the endosome, and are activated by virus derived ligands, such as DNA and ssRNA (6, 52). In common for all of them is that they activate the transcription factor Nuclear factor kappa light-chain enhancer of activated B-cells (NFκB). All TLRs are not expressed by all immune cells, some seem to be restricted in their cellular expression (42). After detection of danger signals from a receptor via PAMP or DAMP there is a cascade of downstream signals, and dependent of receptors involved and the type of ligand, a signaling pathway is activated (42). Signaling via membrane-bound TLRs gives rise to different signaling cascades depending on which TLR is activated. Simplified one could describe the extracellular exposed TLRs as MyD88 dependent activators of the nuclear transcription factor NFκB, where the activation of NFκB leads

to induction of different inflammatory cytokines (46). On the other hand endosomal TLRs signal via TRIF and MyD88 and activates interferon regulatory factors (IRF) and NFκB. Activation of the MyD88 pathway is essentially results in recruitment of IL-1-receptor-associated kinases (IRAK) and downstream the mitogen associated protein kinase (MAPK) and NFκB. The recruited IRAK also, via NFκB essential modifier, removes an inhibitor of NFκB (IκB) (53, 54). Activated NFκB stimulates production of several cytokines, such as IL-12p40 and IL-6. The TRIF pathway does not only activate the NFκB pathway and MAPK, which is activated via TNF receptor associated factor (TRAF) 6 and TRADD, but also activates the TRAF3 signaling to IRF3, leading to IFN -β transcription (42, 55). TLR 7 and 9, the endosomal MyD88-associated TLRs, activates the production of antiviral type I IFN via IRF7. IRF7 is expressed constantly in plasmacytoid DCs, and also IRF5 can be activated via TLR7 and TLR9 (42).

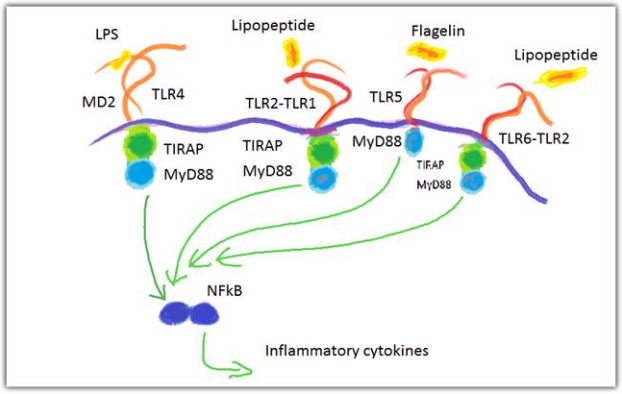


*Figure 3**TLRs on the cell surface.*** The cell surface bound TLRs are specialized in recognition of microbial derived PAMPs, such as flaggelin, LPS and Lipopeptide. These ligands activate a signaling cascade, starting from the adopter-protein TIRAP, via MyD88 to NFκB activation. This results in transcription of inflammatory cytokines.

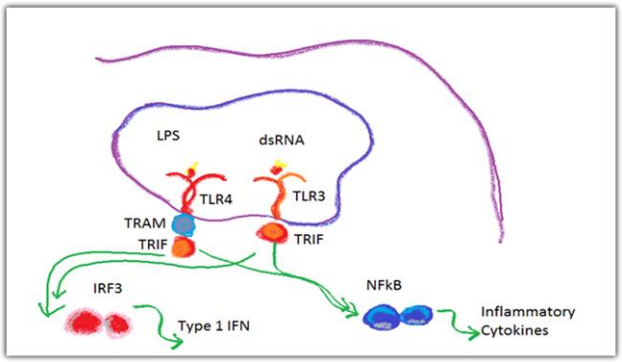


*Figure 4 **The TRIF signaling endosomal TLRs.*** Endosomal TLR4 activated by its ligand LPS signals via the adaptor molecules TRAM and TRIF, in contrast to TLR3, which is activated by dsRNA and signals only via TRIF. However both give rise to activated NFκB inducing inflammatory cytokines, but IRF3 that give rise to type 1 IFNs

1.4.2 Non-TLR receptors of innate immunity

Other receptors of the immune system are C-type lectin receptors (CLR) and NLR. NLR play a pivotal role in activation of the immune complex known as the inflammasome, driving the production of IL-1β and IL-18 (56). CLRs have been demonstrated to be activated after fungal infections, and several members of this protein family have been described (57, 58). However, also immunity against other pathogens than fungi has been described for these receptors. The signaling

of CLR is via immune receptor tyrosine activation (ITAM)-like motifs or ITAM-associated proteins.

1.4.3 **Caspases and the inflammasome**

Caspases constitute a protein family named after their essential function and composition, a cysteine protease with the ability to cleave proteins after aspartic acid residues (56, 59). The caspases have various functions, either inflammatory or apoptotic. Twelve caspases have been identified in humans. Most well studied for immunologists are the caspase-1, with the pro-domain caspase activation and recruitment domain (CARD) – all inflammatory caspases have this domain. Five different protein complexes have been shown to activate caspase 1. These complexes are denoted inflammasome, and their activation pathways involve PAMP or DAMP signaling via different receptors. For example, the NOD, LRR and pyrin domain- containing -inflammasome is activated by anthrax toxin and the NOD-like receptor and pyrin domain containing protein 3 (NLRP3) inflammasome is activated via several DAMPs. Toxins from Salmonella bacteria are detected by the neuronal apoptosis inhibitory proteins (NAIP) and form a complex with NOD-LRR and CARD containing protein 4 (NLRC4) and is therefore named NAIP-NLRC4 inflammasome. One identified caspase that senses DNA has been named absent in melanoma (AIM) 2. The last of the caspase-1 activators described in the literature is the Pyrin inflammasome, activated via bacterial toxins. The effect of Caspase-1 is that it cleaves pro-IL-1β and pro-IL-18, and the protein Gasdermin D, leading to the release of IL-1β and IL-18 (60, 61). Activation of Caspase-1 can occur via the TLR4 pathway or through the non-canonical pathway. Caspase-11 can be activated via LPS in the cytoplasm, and thereafter activates the NLRP3 inflammasome, and this pathway is called the non-canonical pathway (56).

1.5 ALLERGY

Allergy is a hypersensitivity reaction of the immune system towards foreign proteins or glycoproteins. The reported prevalence of any sensitization is reported to be around 30 %, and seems to increase worldwide (62, 63). In this thesis allergy refers to IgE mediated allergy. This is the most common form of allergy, although allergic reactions may also be non-IgE mediated (64). The molecules that the immune system reacts towards are referred to as allergens (64). Individuals prone to produce IgE, due to a personal or familiar predisposition, are referred to as atopic. Clinical manifestation of the immunological reaction of allergy is commonly rhinitis, asthma, skin inflammation or rashes. Systemic reactions occur more rarely and may in worst-case lead to life threatening anaphylaxis (65). The incidence of allergy worldwide seems to increase (63). One proposed reason is the “hygiene hypothesis”. In a simplified way it can be explained by a reduction of Th1 responses against microbes due to less burden of microbes in a modern clean, i.e. hygienic, environment. Thus the Th2 prone immune status of the infant will not deviate to a Th1 promoting status in the absence of microbial challenge, resulting in an increased risk of allergic disease (66). Also lack of regulatory cells and cytokines, like Tregs and IL-10, in a highly hygienic environment is a proposed mechanism of the increased incidence of allergic diseases (66).

1.5.1 **Allergens**

Molecules that cause allergic reaction are referred to as allergens. Allergens are usually proteins, but in rare cases the IgE binding capacity is the sugar residue on glycoproteins. Allergens are suggested to be divided into three subclasses; I) indoor allergens II) pollen allergens and III) plant

and animal food allergens (67). The reason why some proteins are targeted by the immune system with a Th2 type response associated with allergy in unclear. However, several of the indoor allergens derived from house dust mite have been reported to promote Th2 cytokines, or trigger TLR4 activation and induce NFκB of B-cells (67-69). The major cat allergen Fel d 1 and the dog allergen Can f 6 have also been reported to activate TLR4 *in vitro* (70). Cross-reactions, i.e. IgE binding similar epitopes on different allergens or homologous protein might explain why some allergens are IgE binding, but also epitope spreading might explain some sensitization. However a primary sensitization is needed even if this effect could explain why some proteins family have a tendency of being targeted by the immune system (71-73).

1.5.2 **Sensitization phase**

The first encounter of an allergen, does not lead to an allergic reaction. First the immune system needs to promote the B-cells into production of antigen specific IgE. This happens upon presentation of allergen to naïve T-cells, which promote B-cells (figure 5) (74). First the allergens are taken up in the periphery by DCs, which migrates to the lymph node, where the allergen derived peptide can be presented on MHC II to the TCR of naïve T-cells. Theses T-cells differentiates into Th2 cells by IL-4 secreted from the DCs. Exactly how this IL-4 secreting DCs are induced is not fully understood, but one possible explanation is that it might be due to Th2-promoting adjuvant properties of the allergen, and as described above (75, 76). Also other molecules associated to the allergen, such as LPS, could work as an adjuvant and influence the type of response (77). Key molecules in the activation of Th2-responses have been reported to be IL-25, IL-33 and TSPL, molecules released from stressed or damaged epithelium (8, 68, 73, 78, 79). The Th2 cell then activates and promotes the B-cell to undergo class-switch, i.e. change the subclass of antibody production to IgE.

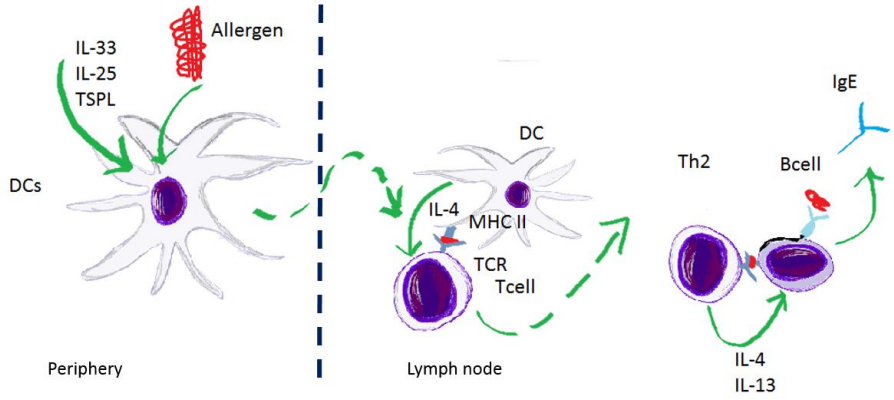


Figure 5 **Sensitization phase** DCs take up the antigen in the periphery, and become activated, IL-4 producing via IL-33, IL-25 and TSPL secreted from tissue. The activated DC migrate to lymph node, and presents the allergen derived MHC II bound peptide to naïve T-cell and activates them via the TCR-MHC interaction and co-stimulatory signaling. The IL-4 producing DC skews the response towards Th2. Th2 cells starts secreting IL-4 and IL-13, and when encountering B-cells presenting the same peptide as before, the T-cell promotes the B-cell to start producing allergen specific IgE.

1.5.3 Effector phase- the immediate reaction

When a sensitized individual, i.e. an individual with pre-formed allergen specific IgE, encounters the allergen an immediate phase reaction occur (74). If the allergen binds to two IgE, that are bound to FcεRI of tissue resident mast cell or circulating basophils, a cross-linking could occur that causes a cell activation (16). This activation leads to release mediators stored in pre-formed vesicles. The content of the vesicles are different immunological mediators (bioactive amines and lipid mediators), enzymes and cytokines. Histamine is one of the key inflammatory mediators, causing bronchoconstriction via smooth muscle constriction, increasing vascular permeability and vasodilatation via its effect on epithelial cells (80). Histamine release, together with other mediators, therefore affects lung function and blood pressure, making breathing more difficult and blood-pressure to drop, as well as causing local edema. The mast cells are also induced to produce and release cytokines, for example IL-4, driving the Th2 skewing even more, as well as other pro-inflammatory cytokines such as IL-5 (81, 82). These reactions occur within minutes after the allergen encounter. The increased permeability and induction of inflammation is causing classical symptoms associated with allergy such as rhinitis, watery and itching eyes, the previous mentioned reduction in airway function, and local swelling and edema (16). The molecules released from the mast cells and basophils also works as chemo attractants for other immunological relevant cells.

1.5.4 Effector phase- the late phase reaction

The late phase reaction occurs after several hours, when the cells recruited to the affected tissue start to exert their effect (74). Here the role of allergen specific Th2 cells is of great importance, for example by secretion of IL-5 that attracts eosinophils (83). Thus not only T lymphocytes infiltrate the tissue, but also eosinophils and to some extent neutrophils. This recruitment increases the inflammatory response (82). Activated eosinophils release inflammatory mediators, propagating and elevating the inflammatory response. Other factors that might play a role in the late phase reaction is FcεRI-IgE dependent antigen presentation (84). The late phase reaction has been regarded as a cause of the remodeling of the airways, leading to chronic and persistent problems (85).

1.5.5 Chronic phase

The chronic phase of allergic inflammation involves the remodeling of the tissue after persistent allergic inflammation (86). For example, in the case of asthma, an increase of mucus producing cells can be observed in the bronchial tract, and inflammation, fibrous tissue and areas of epithelial damage (87). This phase might even increase the risk of respiratory virus infections, due to reduction in the IFNγ response (87).

1.5.6 Allergy to pets

Allergic reactions or sensitization to allergens from furry animals are very common. As an example, in a study made in northern Sweden 2009, the rate of sensitization to cats was 26% and to dogs 25% among the tested population, and a significant increase was observed from 1994 (88). This study was based on skin prick test (SPT). In the same study more than 10 % were sensitized to horse, but no significant increase over time could be detected. The same trend of increased rate of sensitization could be observed in a British study 1996, where 10% of the tested middle-aged men showed IgE towards cat, an increase with 6% since 1975 (89). An association between IgE to pets and asthma has been reported (90).

1.5.7 Pet allergens

The proteins and glycoproteins that humans react towards from pets often belong to the lipocalin family but also other protein families have been described (91). Lipocalins, and serum albumins have been reported to cross-react (92-94). However, no common feature or effect of these proteins explaining why the immune system attack them has been described. The nomenclature of the allergens are based on their Latin name and currently named according to the order they were reported, so the first cat allergen described is therefore called Fel d 1, after *Felis domesticus* 1 (95, 96). Dog allergens are called Can f (after *Canis lupus familiaris*) and horse allergens are called Equ c (*Equinius caballus*). Seven allergens from cat has been characterized, seven from dog and four from horse.

The most dominant allergen from cat is Fel d 1, a protein belonging to the uteroglobin family and found in the saliva and eye. A prevalence of up to 95% among cat sensitized individuals has been reported (97-99). For the cat serum albumin, Fel d 2, the prevalence of sensitization is around 20 % (93, 99). Fel d 3 belongs to the cystain protein family, and only about 11 % of cat allergic patients have been reported to react to this minor allergen (100). Fel d 4 belongs to the lipocalin protein family, with a sensitization rate around 60% (101). Cat IgA and IgM are able to bind IgE and were designated the allergen names Fel d 5 and Fel d 6 (102). Reported prevalence for Fel d 5 is 40%, however no report on the prevalence of sensitization to Fel d 6 is available. The recently identified Fel d 7 and Fel d 8, belonging to the lipocalin family and latherin-like family respectively, have reported prevalence of 17% and 18%, respectively (103).

The major dog allergen Can f 1 belongs to the lipocalin family, as do Can f 2, 4 and 6 (94, 104-106). The prevalence of sensitization is 50%, 25%, 20% and 40%, respectively. The serum albumin of dogs, Can f 3, has a reported prevalence of 20 % (93). The kallikrein protein derived from the prostatic gland of male dogs has been designated Can f 5, with a reported prevalence of 70% (107).

The horse allergen Equ c 1 belongs to the lipocalin family with a reported prevalence of 76% (108, 109). Also Equ c 2 has been reported as a lipocalin protein, but with lower sensitization prevalence and no complete protein sequence reported (110). The serum albumin of horse, Equ c 3, is a minor allergen with a prevalence of less than 10 % (111). A surfactant protein belonging to the latherin family have been named Equ c 4, with a prevalence of 77 % among horse sensitized individuals (112, 113). The previously reported allergen Equ c 5 has been deleted from the IUIS allergen database, however the name is still unavailable for registration of new horse allergens. Table 2 for presents an overview of allergens from dog, cat and horse (106).

Table 2 **Pet allergens** reported allergens from the common pets Dog, Cat and Horse.

Dog allergen	Protein family	Size (kDa)	Reported Prevalence (%)	Source
Can f 1	Lipocalin	23-25	50	Saliva, Dander
Can f 2	Lipocalin	19	25	Saliva, Dander
Can f 3	Albumin	69	20	Saliva, Dander
Can f 4	Lipocalin	16	20	Saliva, Dander
Can f 5	Kallikrein	28	70	Urine
Can f 6	Lipocalin	27-29	40	Saliva, Dander
Can f 7	Epididymal secretory protein	16	17	-
Cat allergens				
Fel d 1	Uteroglobin	14-4	95	Saliva
Fel d 2	Albumin	69	20	Dander, Sera, Urine
Fel d 3	Cystain	11	10	Dander
Fel d 4	Lipocalin	22	60	Saliva
Fel d 5	IgA	400	40	Sera, Saliva
Fel d 6	IgM	800-1000	Not reported	Sera, Saliva
Fel d 7	Lipocalin	17	38	Saliva
Fel d 8	Latherin-like protein	24	20	Saliva
Horse allergens				
Equ c 1	Lipocalin	25	76	Saliva, Dander, Urine
Equ c 2	Lipocalin	17	50	Dander
Equ c 3	Albumin	67	>10	Dander, Sera
Equ c 4	Latherin	17	77	Dander, Saliva
Equ c 5	(deleted from the database)	-	-	-

1.5.8 **Diagnosis of allergy**

There are several different methods to diagnose allergy that physicians rely on. First, important for the physician is the patient's history. This is supported by *in vivo* and *in vitro* diagnostic methods. IgE levels are measured in serum against both allergen extracts and against allergens, i.e. the specific components (114, 115). Common serum-diagnostic methods include ImmunoCAP and the ISAC-chip. Commonly used as well is the SPT, a method when the cellular skin reaction is monitored after the application of a small drop of allergen extract, thereafter puncturing of the dermis, and the resulting wheal is compared to a negative and positive control (116). To investigate the biological relevance of an allergen, an *ex vivo* test of basophil activation has been developed, monitoring the expression of CD63 and CD203c, and when used in clinic referred to as CD-sens (117-119). A test positive for allergen specific IgE is not by itself sufficient to diagnose an individual with allergy. To do so, an association to allergic symptoms has to be made, ultimately an *in vivo* allergen provocation may be performed. An individual with allergen specific IgE is referred to as a sensitized individual.

In clinical setting, SPT is usually based on the use of naturally occurring allergens in the form of allergen extracts. These extract may vary in their content allergen components, and may be hard to standardize, making the amount and content of specific components uncertain (120, 121). However,

with the introduction of novel technologies in the field of allergology, such as recombinant DNA techniques, the possibility to obtain large amount of highly pure allergens has made it possible to improve the diagnosis. The diagnosis has expanded from determining the allergen source to the exact molecule that the individuals have IgE against (122, 123).

1.5.9 **Treatment of allergy**

The easiest to manage allergy symptoms is probably to avoid exposure of the allergen source. Therefore, a correct diagnosis is vital. To complicate matters, some sources such as airborne pollen, might be harder to totally avoid. Some allergen sources are ubiquitous and impossible to fully avoid, such as mites and fungi, thriving in the same habitat as us humans. To these allergens there are symptomatic medicals available, such as antihistamines and corticosteroids, managing immediate symptoms and lowering the allergic inflammation. To handle the risk of anaphylactic reactions the use of adrenalin is recommended, as a self-injectable alternative (124). The development of a humanized anti IgE-antibody, Omalizumab, has created a new treatment regime for e.g. severe food allergies. Not only is the circulating IgE abolished, but the also the mast cell responsiveness is affected by down regulation of FcεRI (125, 126) .

1.5.10 **Allergen-specific immunotherapy**

The only curative treatment for allergic disease today is allergen specific immunotherapy (SIT), where repeated exposure of the allergen is executed in a controlled manner. The first report of SIT is more than 100 years old, however, the exact mechanism of how the effect of SIT is achieved is not fully understood, but apparently the allergen-specific immune response is skewed into non-inflammatory reaction or tolerance (74, 127). The treatment effect seems to be long lasting (128). Several routes of administrations of the allergen (also denoted allergy vaccine) have been developed in addition to classic subcutaneous injections, such as sublingual immunotherapy and intra lymphatic injections (ILIT)(127). Proposed mechanisms of SIT include the induction of regulatory T-cells, and induction of immune suppressive cytokines such as IL-10 and TGF-β (129). IL-10 has been reported to affect both allergen specific IgE production as well as dampening the APCs MHC II presentation and inflammatory Th2-type of response (130-132). Not contradictory to this mechanism, the induction of blocking IgG4 antibodies seems to be another possible mechanism of SIT (133-135). One major downside historically has been the concern of safety and reproducibility of the extracts normally used as allergy vaccines in SIT (136). Although the quality control of the extracts has improved, the development of allergy vaccines produced by recombinant DNA techniques have opened the possibility of individualized therapy with specific allergens (137, 138). The use of recombinant proteins also opens up the possibility of novel approaches modifying the allergens, thereby overcoming safety issues and immunogenic disadvantages of natural proteins (139). These strategies are most commonly targeting the IgE –epitopes in order to make the allergy vaccines safer. One example of this is the use of peptide vaccines which has been tested in clinical trials on cat allergic patients (140, 141). Nevertheless, there is still a risk of systemic activation and anaphylaxis, therefore techniques such as ILIT bear much promise, by reducing the number of injections and also the amount of allergen injected (142).

1.6 VACCINATION

Vaccines are compounds given to individuals to create protection against pathogens (143). The vaccines thus aim to create a protective immune response, which for some pathogens is

achieved by a humoral response, i.e. a B-cell response and protective antibodies, and for others by a cell-mediated response, i.e. a T-cell mediated protection. Vaccines can be given orally, injected in different tissues and even applied on punctured skin (143). They usually consists of attenuated pathogens or non-pathogenic subunits that can be taken up by DCs and presented to the adaptive immunity. The success of vaccines, creating memory to vast part of the population at a modest cost is a great achievement of modern medicine, however some diseases and pathogens are still without a fully protective vaccine treatment.

1.6.1 **Adjuvants**

Adjuvants are compounds that boost the immune response to a specific antigen, creating a stronger response than the antigen alone. Adjuvants are commonly used in vaccination protocols, generating a long lasting immunological memory strong enough to prevent a disease or to clear a pathogen before it becomes dangerous (144). Historically the most used adjuvants are aluminum compounds, such as aluminum hydroxide (Alum) (144). The mechanism of action for of Alum is disputed but several ways to achieve the adjuvant effect have been proposed. One suggested theory was the activation of innate receptor, therefore a MyD88 and TRIF deficient murine model was used, but alum still had an adjuvant effect (145). When alum is injected it forms a depot, and the slow release of antigen has also been suggested as an explanation (146). Lately the importance of the depot effect has also been questioned, since an immunological effect could be detected within the first 2 hours after injection (146, 147). The induction of IL-Iβ and the activation of the inflammasome NLRP3 has recently been proposed as a key mechanism behind the activation following administration of Alum (148). The adjuvant Alum is in particular suited for promoting a humoral Th2 response. In many cases a Th1 type response is however required to obtain protection from disease. Alum is commonly used in SIT (although not in all countries) despite the fact that it is a Th2-promoting adjuvant, which is not favorable in the case of allergy vaccination (149). A lot of research is ongoing to find novel adjuvant candidates that may trigger Th1 responses (144). New types of adjuvants are now being introduced, such as the emulsion MF59 and the TLR4 ligand MPL (144, 150, 151).

1.6.2 **Chitin and chitosan**

Chitin is a naturally occurring carbohydrate polymer consisting of N-acetyl glucosamine, found as a building block in cell-walls of fungi and exoskeleton of arthropods. The polymer can be deacetylated, by treating chitin with a strong alkali solution. The resulting product is chitosan, consisting of N-acetyl glucose amine and glucose amine. Chitosan has been suggested as a potential adjuvant, due to its immune-stimulatory properties and its biodegradability (152). Reports from studies in mice have revealed that chitosan promoted a mixed Th1/Th2 response (153, 154). However, the exact mechanism of how chitosan functions as an immune stimulator is unclear. Previous studies using chitosan have concluded that the inflammasome NRLP3 is activated when cells take up chitosan *in vitro*, in contrast to chitin (155, 156). This activation was reported as dependent of secondary activations signal —such as TLR signaling, and therefore in line with previous findings regarding alum. TLR signaling activates NFκB and upregulate transcription of the cytokine pro-IL-1β.The conversion of pro- IL-1β to IL-1β by Caspase-1, a subunit of activated NRPL3, is considered the key event of the immune-stimulatory effect. Also the release of IL-18 and IL-33 is associated with the activation of the NLRP3 inflammasome. Somewhat contradictory, a NLRP3 and caspase-1 deficient murine models show similar levels of antibodies as wild type mice

after treatment with diesel particles (157). The role of NLPL3 in the immune activation of chitosan is debated and needs further studies. Recently published work shows that chitosan has the ability to promote the cytosolic DNA sensor cGAS-STING pathways, and promote DC maturation and type 1 IFN (158).

1.6.3 **ViscoGel**

ViscoGel is a chitosan-based viscoelastic hydrogel made up by 1% chitosan and 99% water (159). ViscoGel has a randomized pattern of deacetylation, making the chitosan more soluble than conventional chitosan with blockwise deacetylation. Thus, the chitosan used in Viscogel is soluble at physiological pH, making ViscoGel suitable for use in medical applications. The gel can be processed into particles of defined size. Promising results have been reporter from a mouse study were ViscoGel was injected in combination with *Haemophilus influenzae* type b (Act-HIB) vaccine (153). Administration of ViscoGel enhanced the antibody response to Act-HIB. When analyzing the cellular response, the cytokines upregulated indicated both a Th1 and Th2 response, making ViscoGel an interesting adjuvant candidate to study further.

2 AIM OF THE THESIS

The overall aim of this thesis was to develop new approaches to correctly diagnose individuals allergic to dog and horse, and identify the molecules causing sensitization to these animals. This knowledge could be used development of immune modulating therapies. However, today's approved immune stimulating compounds applied in vaccination could be improved, therefore this thesis also investigated a novel adjuvant candidate, the chitosan-based hydrogel Viscogel.

The specific objectives of the thesis were:

I To investigate the allergenicity of dog saliva, and compare it with a commercially available extract based on dog dander.

II To identify new allergens from horse and to evaluate IgE reactivity among horse sensitized individuals to the previously unknown, as well as to known horse allergens.

III To investigate how the chitosan based adjuvant candidate ViscoGel is taken up by antigen presenting cells and mechanisms of cell activation.

IV To evaluate safety and immune stimulating effect of ViscoGel in a clinical phase I/IIa trial, where ViscoGel was evaluated as an adjuvant for the model vaccine Act-HIB.

3 MATERIAL AND METHODS

Methods and techniques used in this thesis are briefly described here. For a more comprehensive description please refer to material and methods in each paper.

3.1 SUBJECTS

In **paper I** sera from dog allergic, or dog sensitized individuals were collected. Sera obtained from these individuals were used to measure IgE against dog dander extract, and dog saliva. Blood samples were taken from patients with dog allergy diagnosis, to perform basophil activation test.

For **paper II**, sera from subjects with positive IgE to horse dander extract (HDE), according to ImmunoCAP test, were collected.

In **paper IV**, in total 120 healthy volunteers of both sexes, age 22-50 years, participated in the two separate parts of the clinical trial.

All studies were approved by the local ethics committee.

3.2 MICE

In **paper III,** C57BL/6 mice were used for *in vivo* evaluation of the local effect after subcutaneous injection of the viscoelastic hydrogel ViscoGel.

This study was approved by the local committee for animal welfare.

3.3 METHODS

3.3.1 Enzyme linked immunosorbent assay (ELISA) (paper I, II, and III)

Two different ELISAs were developed for detecting IgE using monoclonal antibodies to human IgE. In **paper I,** dog dander extract ELISA was validated against IgE values obtained from ImmunoCAP. In **paper II**, an IgE standard was developed using a humanized monoclonal IgE with known kU_A/l value, and used as a reference. IL-1β and IL-18 were measured using commercial ELISA kits in **paper III.**

3.3.2 Flow cytometry (paper I and III)

Cell surface- and intracellular markers were detected with fluorophore conjugated antibodies using multicolor flow cytometry. The chitosan based hydrogel ViscoGel was labelled with FITC and cell uptake analyzed by flow cytometry. Results were analyzed using the FlowJo program.

3.3.3 Confocal laser scanning microscopy (paper III)

Confocal laser scanning microscopy was used to visualize internalized labeled ViscoGel.

3.3.4 Basophil activation test (BAT) (paper I)

To evaluate the degree of degranulation of basophils, whole blood samples from allergic donors were treated with the allergen of interest, followed by analysis of the basophil marker CD203c and surface expression of the granulae marker CD63 by flow cytometry.

3.3.5 THP-1 cells and monocyte derived DCs (MDDC) (paper III)

THP-1 cells with reporter gene for NFκB were differentiated into THP-1 derived macrophages by 72 hours incubation with phorbol 12-myristate 13-acetat (PMA) and MDDCs were differentiated from peripheral blood mononuclear cells (PBMC) using IL-4 and GM-CSF were cultivated and used for investigating uptake of Viscogel, and stimulation of innate responses.

3.3.6 Cellular responses (paper IV)

A cell proliferation assay was applied on PBMC samples to measure the incorporation of [^{3}H]-thymidine, after stimulation with antigens. ELISpot was used to assess the amount of IFNγ producing cells after stimulation with antigen, and a multiplex assay was used to measure cytokines from the cell supernatant after stimulation.

3.3.7 Production of recombinant proteins (paper II)

DNA encoding full-length allergens and adjusted for expression of rare *Escheria coli* codons, were cloned into expression vectors and his-tagged recombinant protein were produced in *E. coli* using a BL21 expression system.

3.3.8 Protein purification (Paper II)

Recombinant His-tagged proteins were purified by immobilized metal chelating chromatography (IMAC) and size exclusion chromatography (SEC). Proteins from natural source were purified with SEC and ion exchange chromatography.

3.3.9 Protein separation (paper I and II)

Electrophoric separation of proteins was conducted using sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE), a method were proteins are separated according to size, and in **paper I** 2D electrophoresis was used to separate protein according to size and iso-electric point.

3.3.10 Immunoblotting (paper I and II)

To detect IgE binding proteins, allergen extracts were separated using SDS-PAGE or 2D electrophoresis, and thereafter transferred to a nitrocellulose membrane and proteins were detected using sera and IgE detection antibodies.

3.3.11 Proteomics (Paper I and II)

Isolated protein bands were subjected to in-gel tryptic digestion and mass spectrometry and database search Blast search was used to identify potential allergens, following Tandem mass spectrometry (MS/MS) analysis, or by using reported peptide sequences.

3.3.12 ImmunoCAP (paper I and II)

ImmunoCAP analyses were performed to measure IgE against the dog dander extract and HDE, and for the selection of IgE negative serum.

3.3.13 Statistical methods (paper I-IV)

In **paper I**, Spearman rank test was used for correlation analysis. In **paper II** linear regression was analyzed for antibody responses. In **paper III** levels of uptake, NFκB, IL-1β and IL-18 were analyzed using the parametric one-way ANOVA and Tukey´s multiple comparison test, after testing for normal distribution. In paper IV, descriptive statistics were used for demographic and safety data, and antibody responses were assessed using analysis of covariance and cellular response were analyzed with Kruskal–Wallis with Dunn's multiple comparisons test and Wilcoxon matched pairs test. $P < .05$ was considered significant.

4 RESULTS AND DISCUSSION

4.1 EVALUATION OF DOG SALIVA AS AN ALLERGEN SOURCE

In **paper I** we investigated dog saliva as an allergen source. Allergy to dog is a common allergy worldwide, affecting between 5-10% of the population (160-162). Seven molecules have been identified as allergens from dog (95, 96). Today's diagnostics and SIT are based on allergen extracts, however the content of allergens in dog dander extract is questioned (120, 163). To our knowledge, no study has previously been done to determine if allergens are present in dog saliva, and to what degree. Therefore we collected saliva from individual dogs, all under sterile conditions to avoid contamination, and a saliva extract was produced.

The dog saliva extract was tested using a validated ELISA, and compared with commercially available dog dander extract using sera from dog sensitized individuals (n=59) (Figure 6). Of the tested sera, 44 showed a positive IgE binding to saliva (Optical density (OD); median, 0.276; range, 0.123–0.891). A portion of the tested sera (23/53; 39%) had a higher IgE reactivity to saliva than to dander, implying that saliva extract contained a different composition of allergens than the dog dander extract, when tested in the validated ELISA. To investigate if the saliva extract could help identifying IgE positivity among subjects with suspected dog allergic symptoms, 55 sera with reported symptoms but with negative IgE value to dog dander extract as measured on ImmunoCAP e5 was tested. In this group 11 had a positive IgE result to the saliva extract (OD; median, 0.139; range, 0.125–0.188). This result further implies the possible role of saliva in sensitization among dog allergic subjects.

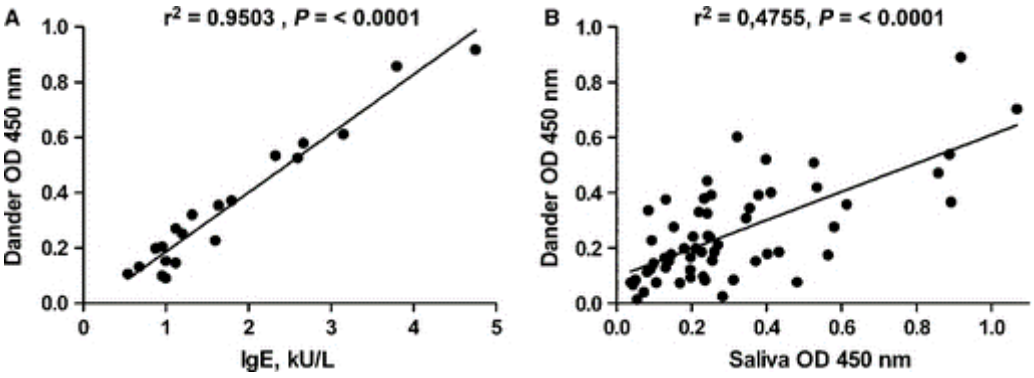


Figure 6 **IgE reactivity of dog saliva**. **A** Correlation between IgE reactivity to dog dander by ELISA and ImmunoCAP results using dog dander extract, where the Y-axis denotes OD value for saliva extract and the x-axis denotes e5 ImmunCAP value (n = 20); (B) correlation between IgE reactivity in ELISA to dog dander extract and dog saliva extract, where the y-axis denotes the dog dander extract O.D. value and the x-axis denotes the saliva extracts OD value., r^2 – correlation factor. (n = 59);

Allergenic activity of the saliva extract was then investigated using the biologically relevant BAT for testing. Three subjects were enrolled to this part of the study (ImmunoCAP e5: 0.1, 2.2 and 2.6 kUA/l). Blood samples were collected and red blood cells were lysed and removed, and the remaining cells were stimulated with dilution series of dog dander extracts and dog saliva extract. All three subjects, with doctors' diagnosis of allergy to dog, showed an up regulation of both tested parameters, CD203c and CD63, however the basophils of one of the tested patients were not reacting to the dog dander extract (Figure 7). This result is in-

line with the previous ELISA finding that some individuals seems to have higher IgE titers, or IgE exclusively to dog saliva.

We also investigated SDS-PAGE of both the extracts, and IgE immunoblotting was performed using a pool of sera from dog sensitized individuals. The protein bands from the SDS-PAGE corresponding to IgE binding bands on immunoblot were cut out and analyzed using the MS/MS and MASCOT software. Can f 1 to 4 and Can f 6 could be detected in the dog dander extract, but no Can f 4 could be identified from the IgE binding bands from saliva. Additionally, four candidate IgE binding proteins were identified. These identified proteins need to be verified, since the possibility of co-migration with other proteins in the gel cannot be excluded.

Individual saliva samples, as well as dander, from different dog breeds were also analyzed against pooled sera (figure 8). We could conclude that a high number of IgE binding bands detected between 14 kDa and 67 kDa from all the dogs. A great individual variation in the patterns of IgE binding could be observed, however a tendency of fewer IgE binding bands could be observed for the tested golden retriever. The sample size in this study is to low, but may indicate that the allergen levels of this dog breed is lower, which has been described before in the literature (164).

In conclusion this study showed that dog saliva may improve the IgE based diagnostics if added as an allergen source when evaluating IgE levels against dog. Moreover, the biologically relevant test, BAT, confirmed that some patients may only react to dog saliva extract.

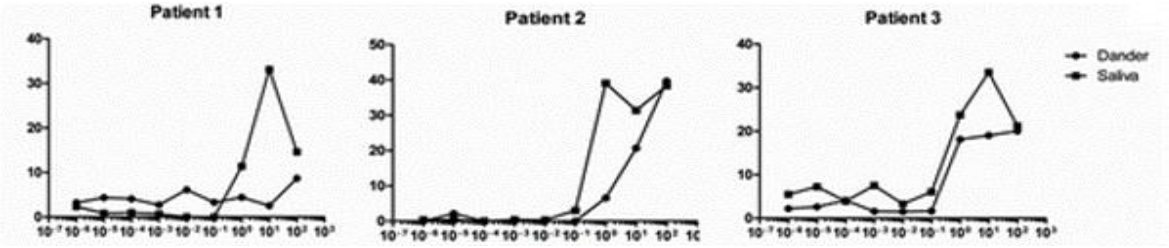


Figure 7 **Basophil activation testing of three dog allergic individuals** Y-axis denotes %-up regulated basophil markers CD 63 and CD203c after activation by dog dander extract or dog saliva extract, and the x-axis concentration in 10 fold serial dilutions, from right to left 10 μg/mL to 10^{-7} μg/ml.

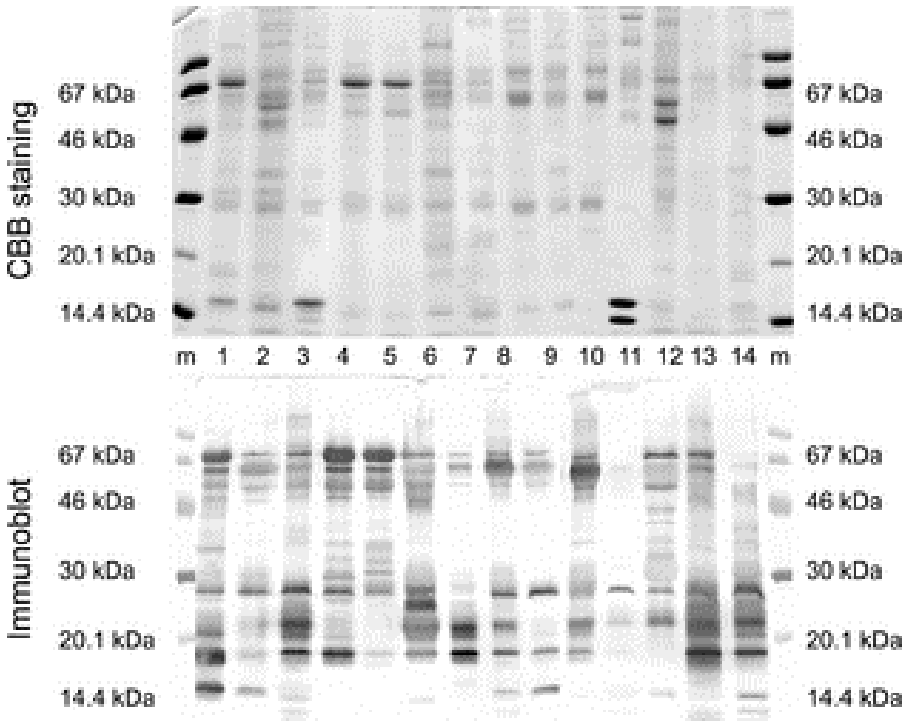


Figure 8 **SDS-PAGE and immunoblot analysis of saliva from different dog breeds visualized with a pool of dog-allergic patients sera.** Lanes indicate m, Molecular weight markers; 1. German Wirehaired Pointer, male; 2. German Shorthaired Pointer, male; 3. German Shepherd Dog, male; 4. Cocker Spaniel, male; 5. Cocker Spaniel, bitch; 6. Doberman Pinscher, male; 7. Doberman Pinscher, bitch; 8. Neapolitan Mastiff, male; 9. Dogue de Bordeaux, male; 10. Saint Bernard's Dog, bitch; 11. Golden Retriever, male; 12. Pekingese, bitch; 13. Mixed breed, bitch; 14. Mixed breed, bitch. Pooled sera from 13 individuals was used.

4.2 IGE PROFILE IN HORSE (EQUUS CABALLUS) SENSITIZED SUBJECTS USING NOVEL AND PREVIUSLY DESCRIBED ALLERGENS

In **paper II** we applied methods similar to the ones in Paper I, but to another allergen source, the horse. First we collected 100 consecutive sera with IgE levels >0.35 kU_A/l against the commercial HDE, ImmunoCAP e3 (HDE-e3). Additionally 50 consecutive sera were collected, as negative controls, with IgE <0.35kU_A/l when tested on ImmunCAP Phadiatope (IgE measurement of 11 allergen sources including HDE), as well as to the main pet allergens rCan f 1, rFeld 1 and rEqu c 1.

An initial immunoblot revealed an IgE binding band of 14 kDa. The corresponding SDS-PAGE band was cut out and subjected to MS/MS protein analysis. The analysis revealed a protein not previously described as a horse allergen, however this protein showed similarity to a previously described allergen from cow (165). This protein was cloned and expressed as a fusion protein in *E. coli*, purified using IMAC and SEC and denoted as Equ c 7. Furthermore, two protein bands of 5 and 10 kDa from SDS-PAGE separation of HDE revealed homology with the previous described major cat allergen Fel d 1 (99) using MS/MS analysis followed by data base search. This protein was designated Equ c 6. Full-length Equ c 2, previously identified as two protein fragments (166), was identified using BLASTP data base search. Recombinant Equ c 2 was expressed and purified. In addition to these three previously not characterized horse allergen candidates, Equ c 1, previously known as a major allergen, was expressed as a recombinant protein and purified, and Equ c 3 and 4 were

purified from HDE and horse serum, respectively. To determine IgE levels in-house ELISA:s with IgE standard was developed.

IgE levels were measured in the 100 consecutive HDE positive subject's sera as well as in sera from the 50 negative subjects. IgE values > 0.35 kU_A/l were considered positive. The rate of sensitization to the novel allergens was 66, 36 and 34 % for rEqu c 2, rEqu c 6 and rEqu c 7 (median 3.38, 9,59 and 1.71 kU_A/l), respectively. Interestingly, several of the negative control sera showed IgE binding to rEqu c 7 (figure 9). When analyzing correlation with the HDE-e3 value only a weak correlation was observed. This might reflect that this allergen is present at an inadequate level in HDE-e3, or possibly be due to cross-reaction to Bos d 3, the cow homologue to Equ c 7, since the selection criteria did not exclude sera from cow sensitized individuals.

rEqu c 2 was produced as a full-length recombinant protein, to our knolledge, for the first time. A considreble portion of the tested positive subjects' sera showed IgE levels against this allergen, and a higher proportion were positive to rEqu c 2 (66 %), than to the major horse allergen rEqu c1 (61 %). Belonging to the lipocalin family, this protein will be intersting to study furter, considering the high degree of cross-reactivity observed among allergens in this protein family (94). For rEqu c 6 34 % of the tested sera showed a positive result, and as in the case for Equ c 2, the posibility to evauate potential cross-reactivity would be of great interest, considering the homology to the major cat allergen Fel d 1 .This is the first time this allergen has been reported in the scientific litrature. nEqu c 4 showed a similar trend among the sera used as negative controll as rEqu c 7, with several clearly positive subjects. Therfore one might draw the same conclusions as for rEqu c 7, i.e. low levels present in HDE-e3 may generate false negative results and thus negatively influencing the selection criteria for the control group.

The previously described allergens evaluated in this study, rEqu c 1, nEqu c 3 and nEqu c 4, together with the two novel allergen candidates Equ c 6 and 7, and the full length Equ c 2, showed the same rate of positive IgE results as the HDE-e3, with 100% of the subjects tested positive to at least one of the allergens (figure 10). To our knowledge, this is the first time components could meet the standards of extract based diagnosics. In conclusion, in **paper II** we evaluated 3 novel full-length horse proteins for their ability to bind IgE in sera from horse sensitized sunbjects. The novel horse allergen candidates identified, add to the list of components that might cause allergic disease and will possibly contribute to improved future diagnostics and SIT.

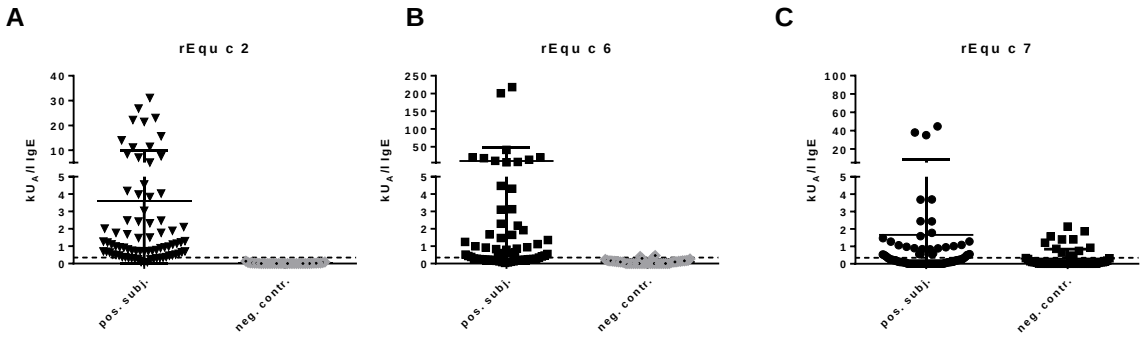


Figure 9 **IgE levels to rEqu c 2, rEqu c 6 and rEqu c 7.** Levels of IgE in sera from 100 HDE-e3 positive subjects and 50 negative control subjects, as measured by quantitative IgE ELISA, against the horse allergens. The Y-axes indicate IgE levels in kU$_A$/l and the dotted line indicates 0.35 kU$_A$/l. Mean value and one standard deviation are indicated.

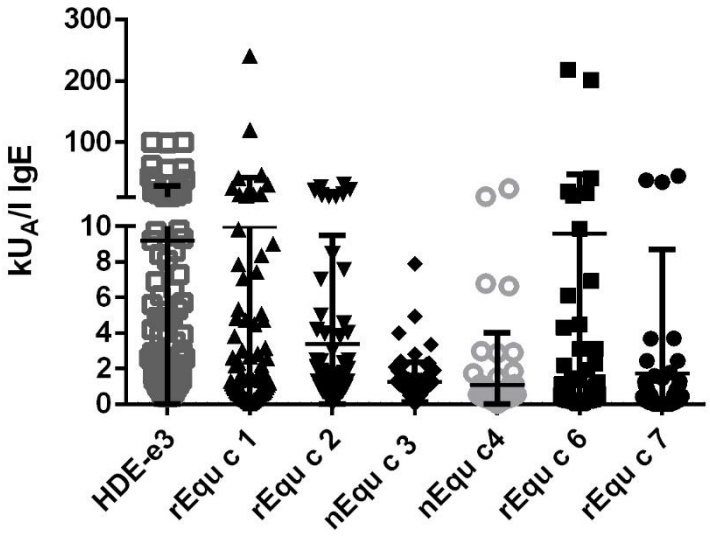


Figure 10 **IgE levels to HDE-e3 and the allergen components rEqu c 1-7.** Serum levels of IgE against the allergen components and HDE-e3 among horse sensitized individuals, X-bar indicates IgE levels in kU$_A$/l , and bar indicate mean value and one standard deviation, n=100.

4.3 UPTAKE OF CHITOSAN-BASED VISCOELASTIC HYDROGEL PARTICLES BY ANTIGEN PRESENTING CELLS AND ACTIVATION OF INNATE IMMUNE RESPONSES

In **paper III** we aimed to evaluate the potential adjuvant ViscoGel, a chitosan-based viscoelastic particulate hydrogel, for its ability to be taken up by APCs and to activate the innate immune system. Chitosan has been proposed as an immune modulator with several properties that are favorable to adjuvant use, such as being biodegradable, non-toxic and to stimulate adaptive immunity, both Th1 and Th2 type responses (153). In terms of allergen vaccination by SIT, such a mixed immune response could be favorable (74). Moreover, the possibility of covalent linking of proteins to the viscoelastic chitosan particles provides a means to prevent systemic spread of the vaccine after injection, addressing safety issues concerning risk for systemic reactions in SIT. Thus chitosan, and ViscoGel in particular,

could be an interesting adjuvant candidate for application in allergy vaccination, as well as in many prophylactic vaccines that have to induce both humoral and cellular immunity to create protection. In order to further investigate the feasibility to apply ViscoGel as an adjuvant and vaccine vehicle, mechanisms of immune activation for the chitosan-based viscoelastic gel particles need to be elucidated. In this study the ability of APC:s to take up and process the viscoelastic chitosan particles was investigated, and furthermore how activation of immune cells is achieved.

We derived macrophage-like cells from the THP-1 cell line, by treating the cells with a stimulator, PMA. Thereafter the THP-1 derived macrophages were stimulated with FITC-labeled viscoelastic particles of two sizes, 10 µm and 200 µm, and the percentage of FITC positive cells was measured using flow cytometry (167). To inhibit uptake cells were treated with the cytochalasin D, a broad inhibitor of uptake acting on actin filaments. Extracellular and intracellular staining was distinguished by quenching the intracellular signal. Particles of both sizes were taken up, and a significant inhibition could be detected following treatment with cytochalasin D, however only a significant reduction of signal could be achieved for particles of 10 µm size. To verify the uptake, cells were visualized using laser scanning microscopy, and z-stack scanning verified internalization of the hydrogel (figure 11). Internalized particles appeared to be around 2-5 µm in size and FITC positive cells could be detected for cells incubated either with 10 µm or 200 µm particles after 24 hours. To further investigate the uptake we differentiated MDDC:s from human PBMCs, and measured the uptake using flow cytometry. These cells were able to readily take up particles in an actin dependent manner.

To investigate the immunological response, the NFκB reporter gene system of the THP-1-blue cell line was used, making monitoring of immune activation possible. The hydrogel was able to activate NFκB to a significant degree when compared to unstimulated cells or cells stimulated with the adjuvant alum. To further analyze if the response could be enhance by engagement of the TLR-4 signaling pathway, cells were pretreated with LPS before incubation with the viscoelastic chitosan particles or alum. No additive effect could be detected in the LPS pretreated cells, either for the viscoelastic chitosan particles or for alum. NFκB triggers transcription of pro-IL-1β, which is processed to active IL-1β via an inflammasome pathway. Therefore IL-1β was measured in cell culture supernatants by ELISA (148). The hydrogel particles induced a significant enhanced IL-1β response in the THP-1 derived macrophages, compared with alum stimulated and untreated control cells (figure 12). Alum was only able to stimulate IL-1β production in combination with a TLR ligand, in this case LPS, and to a significant higher degree than the hydrogel particles, in line with previous reports (148). The hydrogel stimulated did not significantly enhance IL-1β secretion cells in combination with LPS. To support this finding *in vivo*, exudates from mice subcutaneously injected with the viscoelastic hydrogel (200 µm particles) were analyzed after 4 hours and 24 hours post injection, and an up-regulation of IL-1β was detected compared to exudates from exudates from PBS- injected sites. Previous reports on chitosan particles have concluded that the activation pathway is dependent on caspase-1 activity, therefore we

investigated this proposed mechanism using FLICA, a fluorescent compound that binds to caspase-1, and we analyzed the cells using flow cytometry (155, 156). No up-regulation of caspase-1 could be detected for the chitosan hydrogel stimulated cells, either alone or in combination with LPS, thought alum elicited an up-regulation as previously reported.

To conclude, in this study we show that chitosan based viscoelastic particles are taken up by APCs, and that the internalization is actin dependent. In contrast to other reports, we also detected an activation of NFκB and upregulation of IL-1β, and the IL-1β independent of TLR signaling (156).

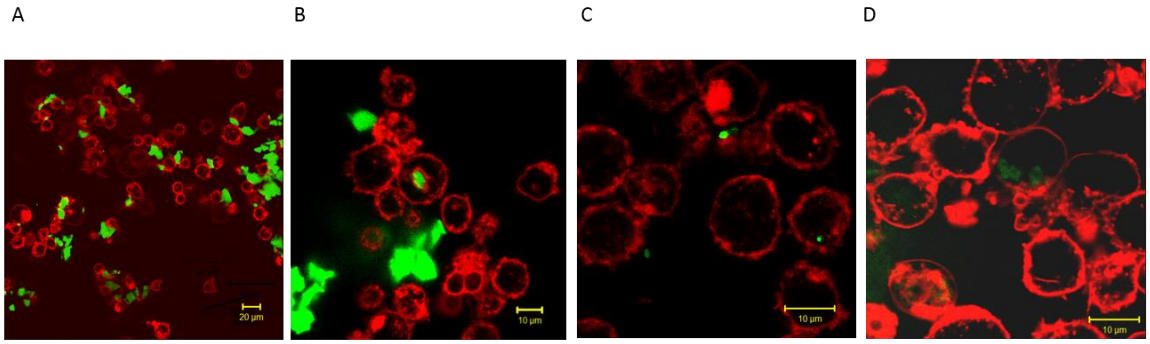


Figure 11 **Uptake of ViscoGel by THP-1 derived macrophages**. **A-D** Analysis by confocal laser scanning microscopy of THP-1 derived macrophages incubated with 10 µm FITC labeled viscoelastic chitosan particels (green) for 2 hours (**A**), 6 hours (**B**) or 24 hours (**C**) and 200 µm particles for 24 hours (**D**). Cell membranes were stained using Cell Mask orange (red). 40x magnification, scale bar indicates size **A** 20 µm and **B-C** 10 µm

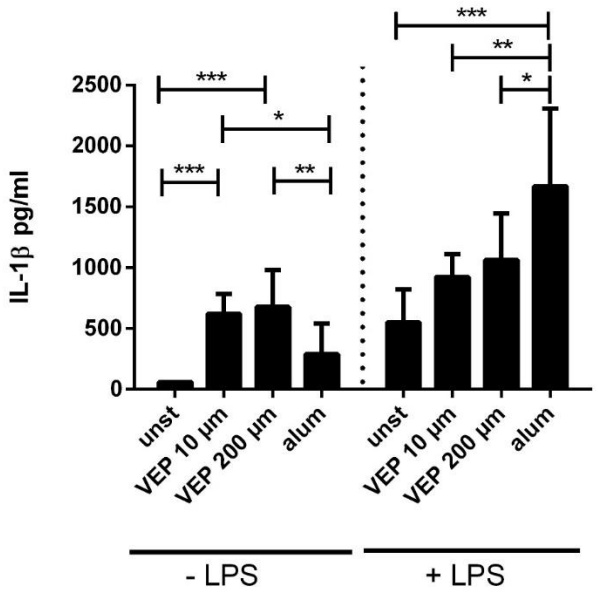


Figure 12 **ViscoGel stimulate the production of IL-1β.** IL-1β measured in supernatants of THP-1 derived macrophages after 24 hours stimulation with ViscoGel of 10 and 200 µm size, and with alum, alone (-LPS) or with the addition of LPS (+LPS). Data presented as mean plus SD. Statistical analysis was performed using one way ANOVA Tukey´s multiple compairsion. The concentration of IL-1 β in cultures of unstimulated cells was set to 62.5 pg/ml, i.e. the detection limit of the assay, since all data points were ≤62.5 pg/ml. *= $p<0.05$, **=$p<0.01$***=$p<0.005$. VEP=ViscoGel particles, unst=cells incubated in medium alone

4.4 EVALUATION OF SAFETY AND EFFICACY OF VISCOGEL IN A SINGLE-BLIND RANDOMISED PHASE I/IIA CLINICAL TRIAL

To study safety and the adjuvant effect of the chitosan particles, i.e. ViscoGel, a randomized phase I/IIa clinical trial was conducted, **paper IV**. Healthy volunteers included in the study were between 22-50 year of age and of both sexes. Subjects included had no reported *Haemophilus influenza* type B (HIB) infection, or contact with individuals with HIB, and had not received previous vaccination against HIB with the vaccine Act-HIB.

In the first part of the study, the primary objective was to investigate the safety of intramuscular injected ViscoGel. This first part also served as dose-finding for the second, phase IIa, part of the study. Three groups with ten healthy volunteers per group received intramuscular injections with Viscogel (gel particle size 200 µm) in escalating doses, 25, 50 or 75 mg (one dose per group). Safety was monitored and adverse events (AE) were registered. A drug safety monitoring board evaluated the result prior to any dose escalation and after completion. The Viscogel was well tolerated at all doses, and no serious adverse events were reported. The highest number of AE:s were observed for the 75 mg group. A majority of reported AE:s was mild to moderate. The dose for the second phase was selected to 50 mg, based on lower incidence of local reaction at the injection site.

In the second part of the trial, efficacy of ViscoGel as an adjuvant was evaluated, as well as the safety. To evaluate the efficacy, ViscoGel was used as an adjuvant for Act-HIB, which was used as a model vaccine. In this part of the study, the participants were randomized to one of five groups with 20 healthy volunteers in each. The subjects were given one intramuscular injection with 0.2 µg Act-HIB with or without ViscoGel, 2 µg Act-HIB with our without ViscoGel, or the standard dose 10 µg of Act-HIB alone. Serum samples were collected at day 0 (prior to injection) and at day 4, 7, 14, 28 and 180 post injection. Act-HIB IgG titers were evaluated using an anti-HIB ELISA validated for clinical use. To evaluate cellular response, whole blood was collected at day 0 and 7 and PBMC were isolated and frozen. After thawing of the cells, cell cultures were stimulated with Act-HIB, tetanus toxoid (TT; as a control for TT present in Act-HIB), or influenza antigen (irrelevant antigen control). The cellular response was assessed by IFNγ ELISpot, cell proliferation and secreted cytokine analyses.

No severe AEs were reported in the second part of the study. A higher incidence of AEs was reported in the groups injected with the combination of Act-HIB and ViscoGel, compared to Act-HIB alone. The primary efficacy was measured as change in IgG levels against Act-HIB at day 28. The highest antibody titers were reported for the 10 µg Act-HIB group, and no significant difference could be detected between the subjects that were injected with a combination of ViscoGel/Act-HIB and those receiving the corresponding Act-HIB dose. To further analyze the IgG result, due to high base-line titers measured at day 0, a subgroup analysis was performed. Subjects were divided according to lower base-line titers, and the anti-Act-HIB titers at day 28 were measured for subjects within the three lower quartiles and the lower half of baseline titers, however no difference between the groups injected with

ViscoGel/Act-HIB could be detected compared to those given the corresponding Act-HIB dose. The cellular response after stimulation with Act-HIB, measured by IFNγ ELISpot, showed a significant up regulation of IFNγ positive cells in the ViscoGel/ 2 µg Act-HIB group at day 7 compared to baseline (day 0), while a significant decrease in the IFNγ response could be detected for two of the groups receiving only Act-HIB, the 0.2 µg and 2µg group (figure 13). To further analyze the cellular response in the groups, PBMCs were stimulated with influenza antigen, and all groups receiving Act-HIB without ViscoGel showed a significant reduction in IFN-γ producing cells at day 7. Interestingly, ViscoGel seemed to counteract this reduction. Secreted cytokines were analyzed supernatants of PBMC-cultures from the 2 µg ViscoGel/Act-HIB and 2 µg Act-HIB groups. A significant increase in IFNγ could be detected at day 7 for the ViscoGel/Act-HIB group. This was observed after in vitro stimulation with both Act-HIB and influenza antigen stimulation. IL-2, IL-4, IL-5, IL-10, IL-17A and TNFα was also analyzed but no change could be detected between day 0 and 7 for these cytokines.

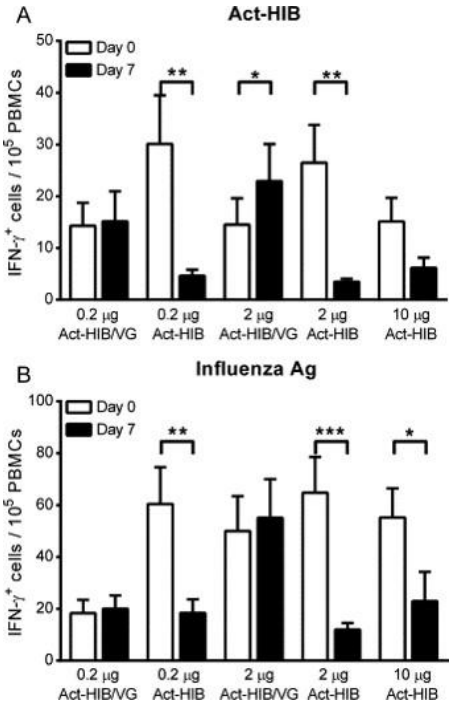


Figure 13 **IFN-γ response analyzed by ELISpot in PBMC cultures in vitro stimulated with Act-HIB** (A) and influenza antigen (B). The number of stimulated IFN-γ producing cells per 100,000 PBMCs at Day 0 (open bars) and Day 7 (black bars) are shown as mean ± SEM of net values where the background IFN-γ producing cell number is subtracted. *p < 0.05, **p < 0.01, ***p < 0.001; Wilcoxon signed rank test. VG denotes ViscoGel

To evaluate proliferation, incorporation of ^{3}H thymidine was analyzed. No change in Act-HIB or influenza antigen stimulated proliferation between day 0 and 7 samples was be observed.

This first-in-man study with the chitosan based hydrogel ViscoGel, showed that ViscoGel is well tolerated in the 70 healthy volunteers subjected to intramuscular injection of ViscoGel. In contrast to previous reports from mice, no effect on the antibody production following

administration of ViscoGel could be observed (153). Compared to a previous Swedish study on anti-HIB titers in the general population, the base-line IgG titers that we found in this trial among subject unvaccinated to Act-HIB were higher than expected (168). This complicated the evaluation of the antibody response in our study. However also the selection of the Act-HIB as a model vaccine might have masked a possible adjuvant effect of ViscoGel, The Act-HIB vaccine is composed of the bacterial polysaccharide conjugated to TT with a good immune stimulatory capacity and a previous study has showed that alum do not increase the anti-HIB responses to such conjugated vaccines (169). The most striking effect of ViscoGel administration was that the chitosan gel seemed to counteract the reduction of the IFN-γ response seen for subjects given the Act-HIB vaccine alone (figure 13). In contrast, subjects that received Act-HIB in combination with ViscoGel displayed a slight up regulation of the IFN-γ response (figure 13).

4.5 ETHICAL CONSIDERATIONS

All of these studies have been conducted in accordance with ethical approval from the Swedish authorities. **Paper IV** describes a first-in-man trial, where all cautions has to be taken that patients may react unexpectedly. **Paper I-III** were all studies performed on human material were conducted *in vitro*, therefore the risk of harming any subjects could be neglected after the blood was collected. All the subject in **paper IV** were healthy volunteers. Personal data were treated anonymous and no results could be tracked to individuals when reported. This is vital for protecting integrity. Following guidelines is of high importance in any research conducted. In particular, long term effects of participating subjects in clinical trials must be carefully considered and the high safety demands have to be met. Also how research is reported in the scientific literature and to the public is vital. The recruitment of volunteers as participants in research are important, and one might argue that research conducted without the patient's best in focus may reduce the voluntary participation in the long run.

5 FUTURE PERSPECTIVES

This thesis focuses on several important aspects on improvement of the management of allergy towards pets, in terms of treatment and diagnostics. It is also valid for the field of adjuvant research in general, examining a novel chitosan based adjuvant candidate. In **paper I** we examined dog saliva as an allergen source, and could show that the saliva extract could identify subjects with possible dog allergy, even though they were negative when analyzing IgE values against dog dander extract. Therefore one might argue that either there are allergens missing from the dander extract that are present in saliva, or the amount of each component is not sufficient enough to be used for detecting allergen specific IgE. One way to overcome such problems could be to spike the dander extract with saliva extract, making a mix of both extracts (170). To address problems with limited amount of several components in the extract, one might argue that there is a need to standardize and quantify allergens present in the extracts and spike the extract with missing components (171).

In **paper I** we also identified four IgE binding proteins not corresponding to known dog allergens. It would be of high interest to evaluate these proteins further, to confirm that they are IgE binding. The identified proteins were BPIFA2, Mucin-5B, ANGPTL5 and the IgA heavy chain constant region. The only of these candidates that has homology with other reported allergens is IgA (102, 172). However, in the case of IgA, which has been described as a pet allergen in cat (Fel d 5), the IgE binding epitope is the carbohydrate galactose-α-1,3-galactose (α-Gal), not the protein per se. The other identified proteins show no striking feature that is common to any known allergen or allergen protein family. If confirmed as IgE binding proteins, they are new dog and pet allergens that should be studied further.

In **paper II** we identified three novel IgE binding proteins from horse. The first one, denoted Equ c 2, was previously only identified as smaller fragment. To further investigate this protein as an allergen, analysis by BAT would be highly interesting to perform. This would allow for testing of the allergenicity in a biologically relevant setting, which is desirable especially as the prevalence of IgE-binding to full length Equ c 2 was higher than the prevalence previously reported for Equ c 1. As Equ c 2 is a member of the lipocalin family, it would be of high interest to examine cross-reactivity with other species, such as dog and cat (92). This is also interesting for the other two allergens identified, denoted Equ c 6 and 7, showing homology with the major cat allergen Fel d 1 and the minor cow allergen Bos d 3, respectively(173, 174). Future aims would also include testing these allergens' ability to activate basophils in BAT. One might also speculate that a possible route of sensitization to Equ c 6 could rather be via cow-horse allergy, with cow acting as the primary source of allergens. This might explain the high prevalence of sensitization among subjects without reported contact to horse (175). The reasons why certain proteins are more prone to cause sensitization and allergic reactions are still debated. Some data have demonstrated that allergens can cause innate immune activation via TLRs (70). Both the Equ c 2 and Equ c 6 are potential activators of TLR4, however human psoriasin (homologue to Equ c 7) can function as a ligand to the PRR RAGE (176). Thus all the novel IgE binding allergens

presented in **paper II** have reported homologues with ability to signal via PRRs, a subject that would be exciting to explore further.

Paper I and **II** both give new insights to the complex pattern of allergen components that individuals might be allergic to. Any expansion of knowledge concerning new allergen components might be beneficial in terms of diagnostics, but also, in the long run, for SIT (177). Several studies using dog dander for SIT have showed that the extracts are not as efficient as for cat (178, 179). Therefore, studies identifying new allergen candidates, as shown in this thesis, may contribute to the development of future personalized diagnostics and treatment by facilitating correct selection of patients according to their reaction profile against specific allergen components.

To evaluate the potential adjuvant properties of ViscoGel, we investigated the chitosan based particles for their ability to be taken up by, and activate APCs (**paper III**). Both sizes, 10 and 200 µm were taken up, and this effect was possible to block using a broad inhibitor of actin dependent phagocytosis. Therefore, to investigate the exact route by which ViscoGel is taken up would be of interest, and if route influences the immunological effect (180). A surprising result was the ViscoGel stimulated up-regulation of IL-1β, without any caspase-1 involvement (181, 182). To speculate, the activation of APCs *in vivo* might be different from *in vitro*. It would be highly interesting to investigate the effect of ViscoGel in IL-1β deficient mice, to elucidate the importance of this activation mechanism. Also of interest is the opportunity to elucidate the importance of variable characteristics of the chitosan included in ViscoGel, e.g. the degree of deacetylation or the net charge of the molecule (156). ViscoGel has a randomized deacetylation pattern, in contrast to other chitosan formulations, with blocks wise deacetylation. To investigate if the net charge of the adjuvant can influence the immune activation, ViscoGel could be a suitable compound for such experiments.

Paper IV gave us the opportunity to study the effect of ViscoGel in humans, for its safety and as a candidate adjuvant. In summary, no significant safety concerns were reported, supporting the application in humans. However, the immune stimulating effect of ViscoGel was not as good as expected, showing no significant enhancement of the antibody response. One might argue that this study detected an up regulation in a Th1 type response, but more striking was the finding that ViscoGel seemed to counteract the reduced IFNγ responses observed when the vaccine Act-HIB was given alone. This makes ViscoGel highly interesting in SIT, to promote a more Th1 type response, or at least brake the dominance of the Th2 response. Not previously addressed in this paper, the ViscoGel manufacturing process allows cross-linking of the chitosan with proteins. This is an advantageous property for application in SIT, by allowing SIT without the risk of systemic spreading of the allergen. ViscoGel could thus be a suitable candidate for improving SIT, and would be interesting to study further in such settings (183).

To conclude, **paper I** and **II** identifies novel allergen sources and components, with potential to improve diagnostics, and immunotherapy, and **paper III** and **IV** addresses the adjuvant

properties of the chitosan viscoelastic gel ViscoGel, showing several promising characteristics for improvement of current SIT protocols.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Allergi är en vanlig sjukdom som upp emot 30 % av befolkningen lider av. Denna sjukdom yttrar sig som en överkänslighetsreaktion mot ett främmande och oftast ofarligt protein. Reaktionen ger upphov till symptom som rinnande näsa och ögon, svullnad och klåda, eksem och pipig andning. Dock kan även allergiska reaktioner leda till allvarligare komplikationer så som astma, och anafylaktisk chock, som är livshotande. Ämnet man reagerar på kallas allergen, och är oftast ett protein. Vanliga allergenkällor att reagera på är pollen, födoämnen som fisk och nötter, kvalster och pälsdjur. Den typ av allergi som behandlas i denna avhandling avser IgE-medierad allergi, som är den vanligaste typen. IgE är en antikropp och de proteiner som IgE binder till definieras som allergen. Denna antikropp kan när den bundit allergen aktivera celler i vävnad eller blod, s.k. mastceller och basofiler. När dessa celler aktiveras så skickar cellerna ut molekyler som skapar den immunologiska överkänslighetsreaktionen. Bland de viktigaste molekylerna för denna typ av reaktion är histamin. Denna reaktion är ofta ganska omedelbar efter exponering. Ytterligare en fas av allergi inträffar senare, oftast flera timmar efter exponering. Första gången man träffar på ett allergen kommer inte kroppen svara med allergisk reaktion, utan först måste cellerna i kroppen ”lära upp” immunsvaret att känna igen allergenet. Denna process när ett allergiskt immunsvar mot allergenet uppstår kallas sensibilisering. Både den s.k. adaptiva delen av immunförsvaret som omfattar antikroppsproducerande celler och immunmodulerande T-celler, och den medfödda (s.k. inat) delen är involverade i sensibiliseringen och avgörande för om allergi uppstår.

För att diagnostisera patienter är patientens historia central för korrekt diagnos. Men även immunologiska tester genomförs, som att mäta förekomst i blod av IgE mot olika allergenkällor. Vanligt förekommande är också så kallat prick-test, då en liten droppe av allergenextrakt appliceras på huden och så punkteras huden, och storleken på rodnaden/svullnaden mäts. Extrakten som används för att diagnostisera pälsdjursallergi är oftast baserade på päls eller hud från allergenkällan. Dessa extrakt har visat sig variera när det gäller allergeninnehåll.

För att undvika allergiska symtom kan man reducera exponering mot källan man blir allergisk mot, men då krävs korrekt diagnos. Övriga behandlingar är symptomatisk behandling, så som anti-inflammatoriska läkemedel och antihistaminer. Utöver det finns det ett biologiskt läkemedel, en antikropp som binder IgE, detta läkemedel ges via injektion. Den enda behandlingen är botande är dock immunterapi, där man traditionellt injicerar små doser av allergenextrakt i ökande mängder så att kroppen tolererar eller byter typ av immunsvar mot allergenet. Exakt vad som är mekanismen bakom denna omprogrammering av immunsvaret är inte helt klarlagt. Denna behandling medför risk att patienten drabbas av systemisk reaktion, med bieffekter som kan vara direkt farliga. Därför utförs denna behandling på sjukhus, kräver försiktig uppdosering och lång behandling med många

injektioner. Immunterapin för allergi använder sig i vissa fall av en hjälpsubstans för att ge ett starkare och bättre immunsvar, dessa ämnen kallas adjuvans. Immunterapi mot allergi skulle kunna förbättras om bättre adjuvans fanns tillgängligt, både när det gäller säkerhet och effektivitet.

Syftet med denna avhandling är att förbättra kunskapen kring allergenkällor och enskilda allergen komponenter som patienter med allergi mot hund eller häst reagerar mot. Detta för att öka möjligheterna till korrekt diagnos och behandling. Vidare utvärderas ett nytt adjuvans med potential att förbättra effekten av immunterapi för att bota allergi.

I **delarbete I** av avhandling undersöks hundsaliv som källa för hundallergener, och hur saliven kan bidra till att ge korrekt diagnos av allergi mot hund. I dag är de extrakt som används baserade på hundmjäll, och det har påvisats att de varierar i hur mycket de innehåller i fråga om allergen. I studien så analyserades IgE nivåerna bland patienter med positivt blodprov mot hundmjäll, och jämfördes med IgE nivåerna mot salivextrakt. Även serum från individer med misstänkt allergi mot hund men med negativt serum-IgE-resultat mot hundmjällsextrakt inkluderades i studien. Dessa uppvisade också IgE mot salivextrakt.

För att undersöka om saliv kan aktivera basofiler, togs ett blodprov från tre individer, varav två med hundallergidiagnos, samt en frisk frivillig. Från detta blodprov analyserades aktiva basofiler. Salivextraktet visades vara aktiverande för de tre testade individerna, men endast för två av individerna med hundmjällsextrakt. För att kunna identifiera möjliga allergener i hundsaliv så genomfördes en proteinseparation och masspektrometrianalys av IgE bindande-salivproteiner. Fyra tidigare okända IgE bindande proteiner kunde identifieras. Dessa potentiella allergen kan vara intressanta att undersöka vidare. I den vetenskapliga litteraturen finns det inget stöd för att vissa hundraser skulle tålas bättre av allergiker, men det hävdas ibland i andra sammanhang att vissa raser är hypoallergena, d.v.s. allergiker tål dem bättre. För att undersöka om hundar kan uppvisa olika profil av allergen, så gjordes en sammanslagning jämfördes saliv insamlat från enskilda hundar tillhörande olika raser och kön med avseende på IgE-bindande proteiner. En stor variation i det individuella mönstret av IgE bindande proteiner hos hundarna kunde påvisas.

Allergi mot häst är det som undersöks i **delarbete II.** Allergi mot häst är förhållandevis vanligt, även bland grupper som inte har någon uppenbar kontakt med hästar. Idag finns fyra kända allergen från häst rapporterade, och dessa benämns Equ c 1 till 4 efter det latinska namnet för häst, *Equus caballus*, och numret efter vilken ordning de upptäckts i. Men i den vetenskapliga litteraturen spekuleras det i om det kan finnas flera okända allergener.

Hypotesen i denna studie var att det finns flera okända allergen som bidrar till allergi mot häst. Ett tidigare inte rapporterat hästprotein visade sig binda IgE när vi undersökte extrakt som används diagnostiskt, och detta identifierades som ett protein med snarlik sekvens till ett känt allergen från ko. Ytterligare ett protein identifierades som visade sig ha likheter med ett vanligt allergen hos katt. Slutligen så identifierades hela molekylen för ett tidigare rapporterat allergen från häst (Equ c 2), men där bara delar av allergenet tidigare varit kända. Samtliga

dessa tre nya allergen producerades i ett baktieriesystem för att möjliggöra analys av IgE-reaktivitet. Dessutom framställdes de tre allergenen Equ c 1, 3 och 4 så att vi kunde jämföra de potentiella nya allergenens betydelse med tidigare kända hästallergen.

Vi undersökte IgE-bindning med en analysmetod som möjliggjorde att vi kunde fastställa mängd IgE i serum med hjälp av en standard. 100 sera från individer med IgE mot hästmjällsextrakt samlades in och deras mängd IgE mot alla de sex allergenkomponenterna analyserades. Alla sera hade IgE mot ett eller flera av komponenterna, och minst 36 % av alla sera hade IgE mot ett av de nyidentifierade allergenerna.

I **delarbete III** undersöktes en ny kitosanbaserad partikulär hydrogel som föreslagits ha immunstimulerande förmåga. Kitosan är en sockerpolymer som tillverkas från kitin, en i naturen vanlig byggsten i olika typer av skelett, t.ex. räkskal. Kitosan kan kemiskt bearbetas till en gel bestående av 1 % polymer och i övrigt vatten. Gelen, som tillverkas under namnet ViscoGel, kan vidare fördelas till partiklar av bestämd storlek.

Det första som undersöktes var effekten av ViscoGel på humana s.k. antigenpresenterande celler. Denna typ av celler har en centralbetydelse vid aktiveringen av ett immunsvar. Cellerna odlades upp och deras förmåga att ta upp hydroelastiska partiklar, vars storlek i denna studie var 10 eller 200 µm, studerades. För denna analys färgades partiklarna in med en färg och undersöktes med mikroskopi och kvantitativt med flödescytometri. Positiva celler, d.v.s. med färg inuti, kunde detekteras för båda partikelstorlekarna med båda metoderna. Aktivering av dessa celler undersöktes också, efter att de odlats i närvaro av ViscoGel. En uppreglering av två centrala molekylär inom immunaktivering kunde detekteras, vilket gör detta partikulära kitosan immunologiskt relevant att fortsätta att undersöka.

I **delarbete IV** beskrivs en randomiserad klinisk prövning av ViscoGel. Detta var en fas I/IIa studie som undersökte säkerhet och effekt av ViscoGel som adjuvans. I den första delen fick 30 friska frivilliga ViscoGel injicerat i muskel. Säkerhet och bieffekter undersöktes för tre ViscoGel doser för att kunna välja en lämplig dos för effektdelen av studien. Ingen patient visade någon allvarlig bieffekt. Efter att dosen valts, så injicerades ViscoGel tillsammans med ett redan beprövat vaccin, Act-HIB, som skyddar mot en bakterieorsakad meningit. För att undersöka effekten så lottades de friska frivilliga till en av fem grupper, en grupp med endast normaldos av modellvaccinet och två grupper med olika mindre mängd vaccin, och två grupper som fick samma doser av vaccinet tillsammans med ViscoGel. För att utvärdera om ViscoGel har förmåga att stimulera immunsvaret mot Act-HIB, d.v.s. verka som ett adjuvans, togs blodprov innan och vid fem tillfällen efter injektionen, och antikroppsnivåerna mot vaccinet analyserades. Ingen skillnad kunde detekteras mellan korresponderande vaccinmängd och vaccinmängd i kombination med ViscoGel. Vid vaccination eftersträvas inte bara ett antikroppsvar utan även ett cellulärt svar. Celler från ett blodprov taget innan och sju dagar efter injektionen undersöktes, och en markant reducering av det cellulära svaret, analyserat med avseende på IFN -γ utsöndring, kunde detekteras bland de individer som inte fått ViscoGel, medan ViscoGel kunde förhindra en reduktion av ett cellulärt immunsvar efter

vaccinering. Sammantaget så tålde deltagarna ViscoGel bra och även om det primära effektmålet inte uppnåddes så är de immunologiska effekterna är av intresse att fortsätta undersöka.

Sammanfattningsvis:
I **delarbete I** så visar vi att hundsaliv är en allergenkälla, och att exponering av hundsaliv bland personer med allergi mot hund kan orsaka reaktion. I **Delarbete II** så identifierades 3 tidigare okända molekyler från häst som kan orsaka allergisk reaktion, vilket kan förbättra diagnostiska möjligheter. I **delarbete III** så undersökte vi att kitosanbaserade partiklar kunde tas upp av immunologiska celler samt aktivera dessa, vilket gör dessa partiklar av intresse för att förbättra vaccin. Slutligen så kunde vi visa i **delarbete IV** att dessa kitosanpartiklar inte orsakade oönskade bieffekter, och kan anses vara säkert att kombinera med vaccin.

To summarize the results from this thesis:
From **Paper I,** dog saliva is a source of allergen, and exposure might lead to allergic reactions among dog allergic patients. In **Paper II,** we identified molecules from horse that can cause allergic reaction, that was previously unknown, possible improve diagnostic methods. In **paper III,** we could conclude that chitosan based particles are taken up by human immunological cells and activate them, making this compound interesting for boosting vaccines. In **paper IV,** we could conclude that the chitosan based particles can be considered safe when injected in healthy subjects in combination with vaccine.

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